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Cell proliferation and survival mechanisms underlying the abnormal persistence of follicular cysts in bovines with cystic ovarian disease induced by ACTH

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ABSTRACT

Cystic ovarian disease (COD) is an important cause of infertility that affects cattle. Alterations in the ovarian micro-environment of females with follicular cysts could alter the normal processes of proliferation and programmed cell death in ovarian cells. Thus, the objective in the present study was to evaluate apoptosis and proliferation in induced ovarian cystic follicles in cows to investigate the follicular persistence. Stage of estrous cycle was synchronized in 10 heifers and 5 were then subjected to the induction of COD by administration of ACTH. After the ovariectomy number of *in situ* apoptotic cells by TUNEL assay, active caspase-3, FAS/FASLG and members of the BCL2 family were compared by immunohistochemistry and multiplex PCR and cell proliferation by evaluation of Ki-67 protein and cyclin D1 and E mRNA. Significantly ($p < 0.05$) lesser proliferative and apoptotic rates were found in cystic follicles from cows with COD compared with those with regular cycles. The relatively minimal proliferation found by immunohistochemistry with Ki-67 marker were confirmed by the gene expression of cyclin D1 and E. Lesser apoptotic rates were associated with decreased amounts of apoptotic-related proteins BAX, FASLG and caspase-3 as well as the *in situ* apoptosis detected by TUNEL assay, and increased amounts of the anti-apoptotic survival factor cellular BCL2 in the cystic follicles of the COD group. The BAX/BCL2 gene expression profile confirmed the immunohistochemical findings. Results from the present study indicate that cellular proliferation and apoptosis are altered in cystic follicles of cattle. The present study provides new insights into the molecular mechanisms underlying the aberrant persistence of follicular cysts and related diseases.

1. Introduction

Cystic ovarian disease (COD) is an important cause of infertility and has been reported in many species including cattle, sheep and pigs. In dairy cattle, COD has been defined as the presence of one or more follicular structures in the ovary/ovaries, at least 20 mm in diameter that persist for more than 10 days in the absence of luteal tissue, interrupting the normal reproductive cycle (Silvia et al., 2002; Peter, 2004; Vanholder et al., 2006). Many factors such as

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stress, nutritional management and infectious disease can coexist in animals with COD; however, the primary cause of this disease has not yet been elucidated. Although the main components of the ethio-pathogenesis of COD are related to the hypothalamus–pituitary–ovarian axis, the persistence of the follicles without ovulation is related to the cellular proliferation and apoptosis mechanisms (Silvia et al., 2002).

In folliculogenesis, the cells that compose the ovarian follicles normally proliferate first and then differentiate. Finally, in non-pathological conditions, cells can take one of two ways: ovulation, if the follicle is dominant in an ovulatory wave (in that case, it finishes with maximum differentiation, which is the transformation in corpus luteum); or atresia, which occurs with most of the non-dominant follicles (Robker and Richards, 1998a,b; Adams et al., 2008).

Cell cycle progression and consequently proliferation are controlled by a balance of positive and negative regulators converging on cell cycle kinase cascades that can be altered by numerous external signals in multiple steps (Robker and Richards, 1998a,b). Cyclins have been identified as positive regulatory subunits of a class of protein kinases termed cyclin-dependent kinases (CDKs). In mammalian cells, at least six different cyclins have been identified, which act at specific stages of the cell cycle. A transient accumulation of cyclin proteins results in the activation of their CDK partners and subsequently in the phosphorylation of target proteins (Zwijsen et al., 1997). D-type cyclins are strongly implicated in controlling progression through the G1 phase of the cell cycle. Three closely related D-type cyclins have been identified in humans (Zwijsen et al., 1997; Yamauchi et al., 2003), and cattle (Zwijsen et al., 1997; Yamauchi et al., 2003), which interact with and activate CDK4 and CDK6, although they have specialized functions in different cell types (Zwijsen et al., 1997; Robker and Richards, 1998a; Yamauchi et al., 2003). In the ovary, cyclin D2 and CDK4 genes are expressed specifically in granulosa cells (Robker and Richards, 1998a), whereas cyclin D1 and cyclin D3 genes are expressed in an overlapping pattern, showing the greatest expression in theca cells (Robker and Richards, 1998b). Cyclin E acts as a positive regulator of cell cycle progression by binding and activating CDK2. It also regulates the G1 to S phase transition (Reed, 1996; Robker and Richards, 1998a).

The main effectors of apoptosis in the ovary are assumed by the FAS system and BCL2 family members (Kim et al., 1999; Roughton et al., 1999). FAS receptor (FAS/CD95) is a member of the tumor necrosis factor (TNF) family, which is activated upon binding the FAS ligand (FASLG), leading to receptor aggregation and the formation of a death-inducing signalling complex (DISC) (Krammer, 1999; Slot et al., 2006). Members of the BCL2 family are considered among the main regulatory proteins acting in the mitochondria. These proteins can be classified into those having either an anti-apoptotic (e.g. BCL2, BclxL, BCL2L2) or pro-apoptotic (BAX, BAD, BIM, BclxS, BOK) function (Slot et al., 2006). The anti-apoptotic proteins block the activation of effector caspases, caspase-3, caspase-6 and caspase-7, which in turn transduce the apoptotic signals (Tilly, 1996). Caspases act at the penultimate stage of cell death (Das et al., 2008), and caspase-3 is functionally required for apoptosis during follicular atresia. The activation of caspase-3 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). Atresia occurs at every stage of follicular development and involves the apoptosis of granulosa cells, oocytes and eventually theca cells (Hsueh et al., 1994; Markstrom et al., 2002).

Cell death *in situ* and the expression of proteins related to proliferation and apoptosis in ovarian cysts has been evaluated from various perspectives. However, most of these studies have been conducted on samples from abattoirs, with the attendant disadvantages of a lack of knowledge of the history of animals (Isobe and Yoshimura, 2000a,b; Isobe and Yoshimura, 2007).

The objective in the present study was to evaluate proliferation and apoptosis in ovarian follicles in an experimental model of COD in bovines compared with cows with regular estrous cycles to investigate the mechanisms underlying follicular persistence. Cell proliferation process by the expression of the proliferation marker Ki-67 and mRNA of cyclins D1 and E were evaluated. In addition, number of apoptotic cells by terminal deoxynucleotidyl transferase-mediated biotin–dUTP nick end labelling assay (TUNEL) and caspase-3, FAS/FASLG and the BCL2 family member were evaluated by immunohistochemistry and PCR multiplex (BAX/BCL2).

2. Materials and methods

2.1. Animals and treatment

All procedures were conducted according to the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science Societies, 1999). Ten nulliparous Argentinean Holstein heifers (18–24 months old; 400–450 kg body weight; maintained under standard husbandry conditions) with regular estrous cycles, previously corroborated by detection of estrus, rectal palpation and ultrasonography, were used. Stage of estrous cycles were synchronized using the Ovsynch protocol in the following manner: the animals were injected with a gonadotropin-releasing hormone (GNRH) (Receptal®, Intervet, Argentina, 4 μg/ml, 5 ml/animal) on Day 0, prostaglandin $F_{2\alpha}$ (Iliren®, Intervet, Argentina, 0.2 mg/ml, 5 ml/animal) on Day 7 and GnRH on Day 9. Observation for estrus began 24 h after the second GnRH treatment and was performed every 12 h. The time of the first detection of estrous behavior was designated Day 0 of the estrous cycle (Gumen et al., 2003).

Beginning on Day 15 of a synchronized estrous cycle, five heifers received subcutaneous injections of 1 mg/ml, (1 ml/animal) tetracosactrin hexaacetate (Synacthen Depot, Novartis, Basel, Switzerland), a synthetic polypeptide with ACTH activity, every 12 h for 7 days. Five control animals received saline (1 ml) (Dobson et al., 2000; Ortega et al., 2008). Ovarian ultrasonographic examinations were performed in all animals as previously described using a real-time, B-mode scanner equipped with a 7.5 MHz, linear-array, intrarectal transducer (Aloka,

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

Antibodies	Clone/source	IHC antigen retrieval	Dilution
Primary antibodies			
Ki-67	Clon BGX-Ki67. Biogenex (San Ramon, CA, USA)	Citrate Buffer pH 6. Microwave	IHC: 1:30
Caspase-3	Polyclonal. AF835. R&D systems (USA)	–	IHC: 1:600 WB: 1:600
BAX	Polyclonal. PU347-UP-Biogenex (San Ramon, CA, USA)	–	IHC: 1:30 WB: 1:500
BCL2	Polyclonal. ab 7973. Abcam (Cambridge, MA, USA)	–	IHC: 1:100 WB: 1:100
FAS	Clon CH11 Millipore. (Billierica, MA, USA)	Buffer Citrate pH 6. Microwave	IHC: 1:350 WB: 1:400
FASLG	Polyclonal. ab2440. Abcam. (Cambridge, MA, USA)	–	IHC: 1:50 WB: 1:100
Secondary antibodies			
Biotinylated-anti-rabbit IgG	Goat Polyclonal. 65-6140-Zymed (San Francisco, CA, USA)	–	1:100
Biotinylated-anti-mouse IgG	Goat Polyclonal. AP181B Chemicon (Temecula, CA, USA)	–	1:100
Anti-mouse IgG peroxidase	Goat Polyclonal. (Amersham, Buckinghamshire, UK)	–	1:500
Anti-rabbit IgG peroxidase	Goat Polyclonal. (Amersham, Buckinghamshire, UK)	–	1:1000

SSD 500; Wallingford, CT, USA) (Sirois and Fortune, 1988). Measurements were made on a single frozen image of the apparent maximal area of each follicle using the average diameter in two directions at right angles to each other. All ultrasonic examinations were performed by the same operator. The growth and regression of follicles >5 mm, corpora lutea and cysts were monitored. Daily ovarian ultrasonography was performed through a complete estrous cycle (21–23 days in the control group) and from Day 14 (Day 0 = day of estrus) until Day 48 (treated group). Cysts detected by ultrasonography were defined as any follicular structure of 20 mm or greater in diameter that was present for 10 days or more without ovulation and corpus luteum (CL) formation (Dobson et al., 2000). The first day of cyst formation was the day a follicle attained 20 mm or more in diameter and the ovaries were removed 10 days later by flank laparatomy. In the heifers used as a control, an ovariectomy was conducted when the dominant follicle reached a diameter of >10 mm, in the absence of an active CL, to obtain normal growing follicles (approximately Day 18). Blood samples were centrifuged at 1000 *g* for 30 min and serum was stored at −20 °C until hormonal analysis was performed [data not shown; parallel studies; Ortega et al., 2008].

2.2. Tissue sampling and classification of follicles

During the dissection of the ovaries, the follicular diameter was measured with callipers and follicular fluid from each follicle was aspirated and stored separately at −20 °C. For the RT-PCR only small (<5 mm) and large (>10 mm) healthy tertiary follicles from control animals and cystic follicles (>20 mm) from COD animals were used (Parrott and Skinner, 1998; Silvia et al., 2002). Follicles of different diameters of each group were immediately frozen at −80 °C until used in gene expression assays and western blotting for the determination of the specificity of the antibodies used in immunohistochemistry. Follicular health status was confirmed by hormonal concentrations in follicular fluid [data not shown; parallel study; Ortega et al., 2008].

Tissue remained of the ovaries after taking the samples using molecular biology techniques, were fixed in 4% buffered formaldehyde for 8 h at 4 °C and then washed in phosphate buffered saline (PBS). For light microscopy, fixed tissues were dehydrated in an ascending series of ethanol, cleared in xylene and embedded in paraffin. Thick sections (5 μm) were mounted on slides previously treated with 3-aminopropyltriethoxysilane (Sigma-Aldrich, St. Louis, MO, USA) and primarily stained with haematoxylin–eosin for a preliminary observation of all ovarian structures (Salvetti et al., 2004). For the immunohistochemical and *in situ* detection of apoptosis, follicles were classified into the following groups: secondary, tertiary, atretic; and cystic follicles (Silvia et al., 2002). Secondary follicles were composed of one oocyte surrounded by two or more granulosa cell layers, without an antrum. Tertiary follicles were composed of one oocyte surrounded by a stratified epithelium of granulosa cells (cumulus oophorus) with the follicular antrum present and with a diameter of up to 10 mm. Tertiary follicles containing less than 1% apoptotic cells were regarded as vital tertiary follicles, whereas follicles containing more than 1% apoptotic cells were classified as atretic (D'haeseleer et al., 2006). Only cystic follicles with a complete granulosa cell layer were used.

2.3. Immunohistochemistry

The details, suppliers and concentrations of antibodies used are reported in Table 1. Each antibody was assayed in five sections (minimum) of each ovary from each heifer. A streptavidin-biotin immunoperoxidase method was performed as previously described (Ortega et al., 2009; Salvetti et al., 2004). Briefly, after deparaffinization, microwave pre-treatment (antigen retrieval) was performed by incubating the sections in 0.01 M citrate buffer (pH 6.0). The endogen 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. All sections were incubated with the primary antibodies for 18 h at 4 °C and then for 30 min at room temperature with biotinylated secondary antibodies selected specifically for each of the

Table 2
Primers sense and antisense for bovine BCL2, BAX, cyclin D1 and cyclin E and PCR conditions.

Gene	Primer sequences	cDNA (pb)	PCR conditions	Reference
Cyclin D1	Sense 5′-TGGAGCCCCTGAAGAAGAGC-3′ Antisense 5′-GGAGGGTGGGTTGGAAATGA-3′	332	94, 55, 72 °C 33 cycles	Yamauchi et al. (2003)
Cyclin E	Sense 5′-TTCTGGATTGGCTGATGGAG-3′ Antisense 5′-AAGCAGCGAGGCACCATAA-3′	462	94, 52.4, 72 °C 33 cycles	Yamauchi et al. (2003)
BCL2	Sense 5′-ATGTGTGTGGAGAGCGTCAA-3′3′ Antisense 5′-CAGACTGAGCAGTGCCTTCA-3′	201	Multiplex Bcl-2 + Bax: (56 °C, 30 s) × 6 cycles, (58 °C, 30 s) × 10 cycles, (60 °C, 30 s) × 12 cycles, [60 °C (−0.5 °C/cycles)] × 6 cycles, (57 °C, 30 s) × 6 cycles, (56 °C, 30 s) × 6 cycles, (55 °C, 30 s) × 10 cycles	Colitti et al. (2004)
BAX	Sense 5′-TGGGTGTCCCAAAGTAGGAG-3′ Antisense 5′-CACCCTCAAGATTGTCAGCA-3′	431	94, 52, 72 °C 31 cycles	Shibaya et al. (2007)
GAPDH	Antisense 5′-GGTCATAAGTCCCTCCACGA-3′	103		

two types of primary antibodies used (mono- or polyclonal). The visualisation of the antigens was achieved by the streptavidin-peroxidase method (BioGenex, San Ramon, CA, USA), and 3.3-diaminobenzidine (Liquid DAB-Plus Substrate Kit; Zymed, San Francisco, CA, USA) was used as the chromogen. Finally, the slides were washed in distilled water and counterstained with Mayer's haematoxylin, dehydrated and mounted.

To verify immunoreaction specificity, adjacent control sections were subjected to the same immunohistochemical method, replacing primary antibodies with rabbit and mouse non-immune sera. The specificity of the secondary antibodies was tested by incubation with primary antibodies raised against human antigens with a proven negative reaction to tissues of cattle: anti-CD45 (Clon: PD7/26; Dako, Carpinteria, CA, USA) and anti-Ki-67 (polyclonal, rabbit anti-human Ki-67; Dako, Carpinteria, CA, USA). To exclude the possibility of non-suppressed endogenous peroxidase activity some sections were incubated with DAB reagent alone.

2.4. Identification of apoptotic nuclei by TUNEL assay

Apoptotic nuclei were identified using the ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon, Temecula, CA, USA) according to the manufacturer's protocol. Endogenous peroxidase was blocked by immersing slides in 3% hydrogen peroxide (v/v). Negative controls were treated in the same manner except that the TdT labelling enzyme was omitted (Gavrieli et al., 1992). Apoptotic nuclei were visualised with DAB as the chromogen substrate (Biogenex, San Ramon, CA, USA) and counterstained with Mayer's haematoxylin. Cells showing dark brown staining from the colorimetric reaction were considered positive for DNA fragmentation (Huppertz et al., 1999).

2.5. Western blotting

To test the specificity of the antibodies that detect apoptotic-related proteins, healthy, atretic and cystic follicles from the ovaries of cattle were homogenized in a radio-immunoprecipitation assay lysis buffer consisting of 1% (v/v) IGEPAL CA630 (octylphenyl-polyethylene glycol), 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 50 mM sodium fluoride (all from Sigma-Aldrich Corp., Brooklyn, NY, USA), 0.1 M PBS and a protease inhibitor cocktail (Complete Mini Protease Inhibitor Cocktail Tablets, Roche, Mannheim, Germany). Ovarian homogenates were centrifuged at 14,000 rpm for 20 min and supernatant stored frozen at −80 °C. Proteins (40 μg) were separated by SDS-PAGE (15% resolving gel). Proteins were transferred onto nitrocellulose membranes (Amersham, Buckinghamshire, UK), blocked for 1 h in 2% non-fat milk in Tris-buffered saline (TBS) containing 0.05% Tween-20 (Sigma-Aldrich Corp., Brooklyn, NY, USA), and then incubated overnight at 4 °C with specific primary antibodies (Table 1). Following washing, membranes were treated for 1 h with corresponding secondary peroxidase-conjugated antibody (Table 1). Immunopositive bands were visualzied with a chemiluminescent

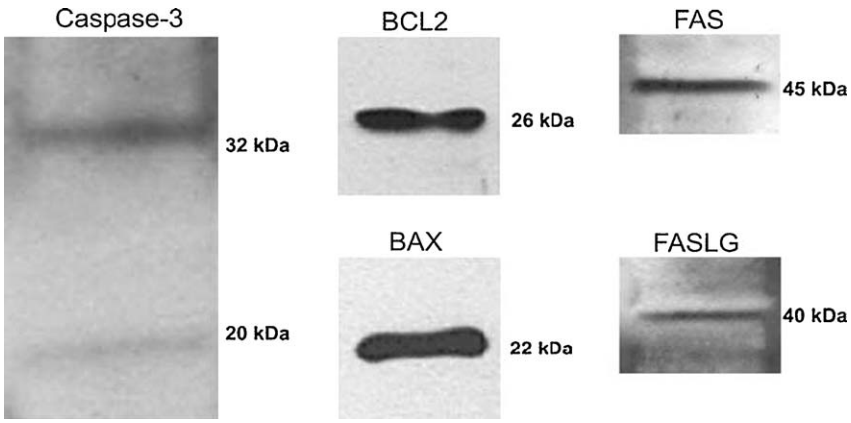

Fig. 1. Verification of antibody specificity by western blot analyses of ovarian homogenate for pro- and anti-apoptotic proteins in cattle.

detection kit (ECL-Plus, GE-Amersham, Buckinghamshire, UK).

2.6. RNA isolation and RT-PCR assays

2.6.1. Total RNA extraction

Total RNA was isolated from the wall of small or large healthy tertiary follicles and cystic follicles using Trizol LS reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions with slight modifications. Briefly, 50–100 mg of frozen tissue was homogenized with 750 ml of Trizol LS reagent and incubated for 5 min at 4 °C. 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 with an equal volume of isopropanol for 60 min at −20 °C and centrifuged at 12,000 × *g* to obtain the pellet of mRNA that was then washed and centrifuged at 1000 × *g* with 75% ethanol for 10 min at 4 °C. Alcohol was replaced by diethylpyrocarbonate (DEPC; Sigma-Aldrich Corp., Brooklyn, NY, USA)-treated water pre-warmed at 55–60 °C. To quantify the amount of total RNA extracted, the optical density (OD) at 260 nm was determined, after which samples were aliquoted and stored at −80 °C until further use. The total RNA integrity was verified electrophoretically on a 1% agarose gel (w/v) and by measuring the OD of the OD260/OD230 nm absorption ratio.

2.6.2. RT-PCR

To avoid putative genomic DNA contamination, RNA samples were treated with DNAse (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. Single-stranded cDNA was reverse transcribed from total RNA DNAse treated with a master mix (MMLV buffer, DTT, RNA out, M-MMLV reverse transcriptase, dNTP) and random primers (Invitrogen, San Diego, CA, USA). The reverse transcription conditions was undertaken at 10 min of annealing at 25 °C, 50 min of cDNA synthesis at 37 °C and 15 min of inactivation at 70 °C.

The sequences of the oligonucleotide primers were synthesised by Invitrogen (San Diego, CA, USA) from published reports. To confirm the integrity of the mRNA templates and the RT-PCR protocol, the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal standard was examined in all samples. The sizes of amplified fragments are listed in Table 2. To perform semi-quantitative PCR, aliquots of cDNA samples equivalent to 10 ng, previously quantified by the Qubit method (Invitrogen, San Diego, CA, USA), were amplified in a standard thermocycler (Techne TC-312, Stone, UK). Each reaction mixture contained: 2 U Taq polymerase (5 U/μl, Invitrogen, San Diego, CA, USA), 0.75 μl $MgCl_2$ (50 mM), 0.2 μl dNTP mix (25 mM) and 0.625 μl of each primer (20 μM) in a final volume of 25 μl of 1× PCR Taq buffer. There were three replicates of each sample. As a negative control, water was used instead of cDNA for the PCR reaction mixture to exclude any contamination from buffers and tubes. This PCR protocol represents optimised conditions and linear phase amplification for each of the primer sets employed. Amplification conditions for each gene are detailed in Table 2.

RT-PCR products were resolved in 2% (w/v) agarose gels, stained with GelRed™ (Biotium, CA, USA) and photographed under UV transillumination (Labnet, Edison, NJ, USA). Their molecular sizes were then determined by comparison with DNA standards (Ladder 10pb, PB-L Productos Bio-Lógicos, Buenos Aires, Argentina).

2.7. Image analysis

Image analysis was performed using the Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver Spring, MA, USA). For the immunohistochemistry technique, images were digitized using a CCD colour video camera (Motic, 2000, Motic China Group, China) mounted on a conventional light microscope (Olympus BH-2, Olympus Co., Japan), using an objective magnification of 40×. 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 inter-assay controls to maximise the levels of accuracy and robustness of the method (Ranefall et al., 1998).

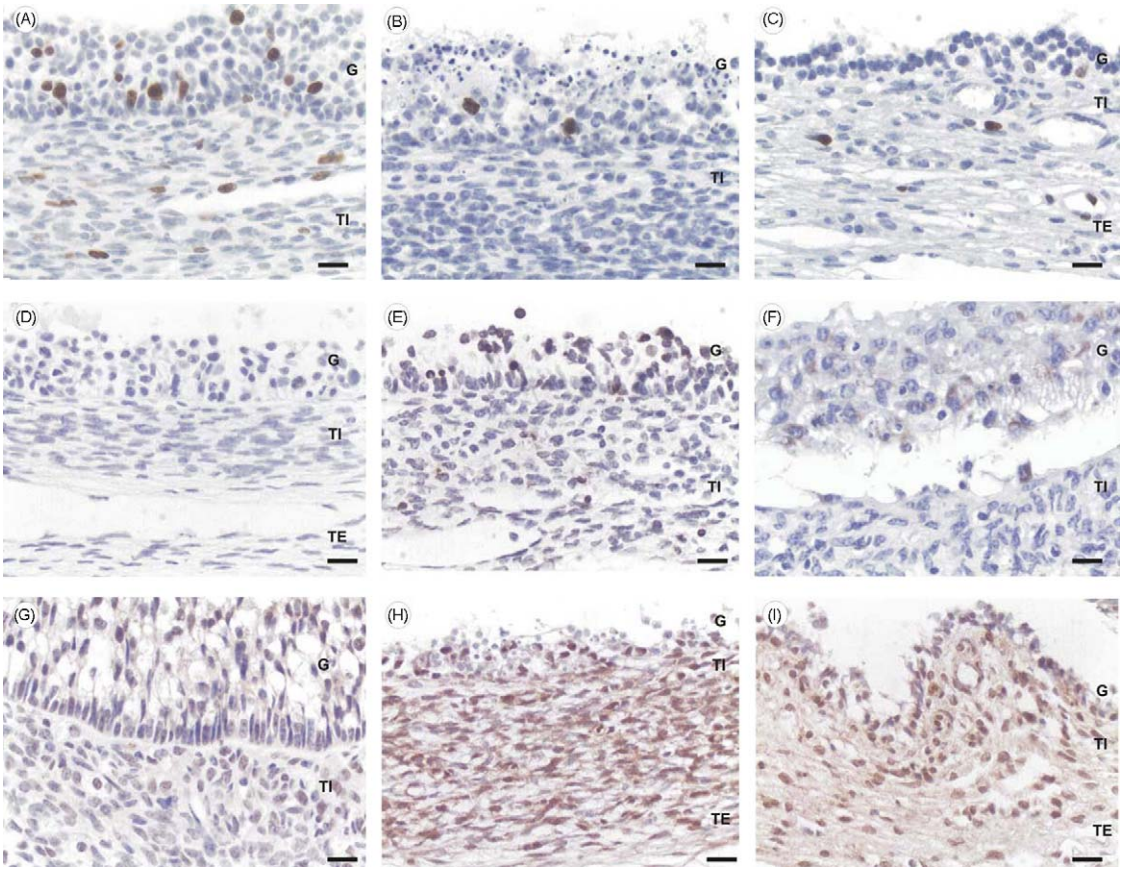

Fig. 2. Localization of Ki-67, FASLG and caspase-3 by immunohistochemistry and *in situ* apoptosis by TUNEL. Positive label is a brown staining of the cytoplasm/nucleus of the cells. (A), (D) and (G) correspond to healthy tertiary follicles of control animals; (B), (E), (H) and (F) correspond to atretic follicles of control animals and (C) and (I) correspond to cystic follicles of cows with COD. (A–C) Ki-67 immunolocalization, (D–E) TUNEL, (F) FASLG and (G–I) caspase-3 immunolocalization. Bars = 20 μm.

The methodological details of image analysis as a valid method for quantifying have been described previously (Baravalle et al., 2007; Salvetti et al., 2007; Ortega et al., 2007a,b, 2009). The major strength of the imaging approach used in this study is the visualisation of the *in situ* localization of proteins within cells of interest. In the past decade, computerized image analysis systems have been developed to obtain objective and accurate quantification of biological markers (Lejeune et al., 2008; Ortega et al., 2006, 2008, 2009).

The image analysis score was calculated separately in each follicular wall layer (granulosa theca interna and theca externa) from at least 50 images of the secondary, tertiary, atretic and cystic follicles from ovaries of both groups.

TUNEL and Ki-67 staining were evaluated by counting positive cells/total cells for each layer to obtain an index of positive cells. The percentage of the immunohistochemical-stained area (IHCSA) was used for the determination of caspase-3, BAX, BCL2, FAS and FASLG immunohistochemistry. The IHCSA 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 colour (brown stain). The brown stain was selected with a sensitivity of 4 (maximum 5) and a mask was then applied to separate the colours permanently. The images were then transformed to a bi-level scale TIFF format.

Agarose gel images were digitised using an Olympus digital camera and PCR products analysed using the Image Pro-Plus 3.1 program. GAPDH mRNA was selected as an internal control because the GAPDH mRNA remained constant in all experimental conditions. In comparative PCR analysis, the absolute OD values for each PCR product were obtained by densitometry and were normalized with amounts of GAPDH. Relative amounts of the specific mRNA were expressed in arbitrary units.

2.8. Statistics

The number of individuals per group was obtained from a sample size calculation that evaluated the number of individuals necessary to produce an estimate of the immunoreactivity that would fall within 0.4 units of the real value. A statistical software package (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA) was used to perform the statistical tests. The differences between the groups of data were assessed by one-way ANOVA, followed by Duncan's multiple range tests. $P < 0.05$ values

Table 3
Immunohistochemical analysis of different proliferation and apoptotic proteins and DNA fragmentation in granulosa cells from cows of control and COD groups.

	Ki-67*	TUNEL*	CASP3**	BCL2**	BAX**	FAS**	FASLG**
C							
SF	17.17 ± 8.84^{bc}	0.00 ± 0.00^{a}	2.37 ± 2.85^{a}	14.51 ± 8.90^{acd}	8.77 ± 4.51^{a}	17.16 ± 6.92^{a}	0.00 ± 0.00^{a}
TF	36.89 ± 7.32^{a}	0.00 ± 0.00^{a}	2.61 ± 1.46^{a}	18.62 ± 6.78^{cd}	11.03 ± 4.40^{a}	16.14 ± 6.68^{a}	0.00 ± 0.00^{a}
AF	6.33 ± 3.67^{c}	8.25 ± 4.50^{b}	10.06 ± 4.10^{bc}	2.01 ± 2.60^{b}	47.56 ± 1.97^{b}	29.61 ± 5.67^{b}	5.51 ± 1.61^{b}
COD							
SF	24.33 ± 10.07^{ab}	0.00 ± 0.00^{a}	4.84 ± 3.47^{ab}	12.44 ± 5.95^{abc}	7.65 ± 3.80^{a}	16.50 ± 8.88^{a}	0.00 ± 0.00^{a}
TF	13.50 ± 4.80^{bcd}	0.00 ± 0.00^{a}	3.46 ± 3.60^{a}	17.34 ± 10.47^{cd}	9.91 ± 2.58^{a}	15.48 ± 10.70^{a}	0.00 ± 0.00^{a}
AF	1.25 ± 0.96^{d}	21.50 ± 11.02^{c}	14.10 ± 7.61^{c}	5.45 ± 1.93^{ab}	28.54 ± 13.19^{c}	29.44 ± 4.56^{b}	2.50 ± 2.73^{b}
CF	1.25 ± 1.50^{d}	0.00 ± 0.00^{a}	7.02 ± 4.84^{ab}	20.12 ± 3.10^{cd}	12.26 ± 4.90^{a}	30.37 ± 9.27^{b}	0.00 ± 0.00^{a}

The values represent mean ± standard deviation. Values in the same column with different superscripts (a–d) differ ($p < 0.05$). *Abbreviations*: C: control; COD: cystic ovarian disease; SF: secondary follicles; TF: tertiary follicles; AF: atretic follicles; CF: cystic follicles; CASP3: caspase-3.
* Percentage of positive cells.
** IHCSA: immunohistochemical-stained area.

were considered significant. Results were expressed as mean ± SD.

3. Results

3.1. Experimental model

The successful induction of COD was confirmed by the ovarian morphology and hormone concentrations. In treated animals, healthy developing follicles, follicles showing different degrees of atresia and one large cyst (in one ovary) with a complete granulosa cell layer were observed. CL were absent in all cases. Ovaries from control animals exhibited follicles in various stages of development including primary, secondary and tertiary follicles, as well as atretic follicles and CL. In addition, the induction was confirmed by serum and follicular fluid hormone analysis (data not shown).

3.2. Antibody specificity

The results from western blot analyses of ovarian homogenate are summarized in Fig. 1. Western blot analysis only detected intense positive bands of appropriate sizes for each of the molecules studied (caspase-3, BCL2, BAX, FAS and FASLG). The BAX antibody detected a single band at 22 kDa, BCL2 was detected as a single band at approximately 26 kDa, FAS antibody detected a band at 45 kDa and FASLG was observed as a band at 40 kDa. Although the manufacturer's specifications indicated that the antibody only identified active caspase-3, two bands were observed in the immunoblot: a band corresponding to the clivated caspase-3 at 20 kDa and a band corresponding to the pro-enzyme (32 kDa).

3.3. Cell proliferation analysis

The proliferation index was evaluated by Ki-67 immunohistochemistry. Granulosa cells of healthy tertiary follicles from the control group (Fig. 2A) had a greater proliferation index than the tertiary, atretic and cystic follicles from COD group and secondary and atretic follicles from control group (Table 3). Secondary follicles from the COD group exhibited an elevated proliferation index (Table 3). In both thecae, tertiary follicles from the control group had a greater proliferation index with the lesser index in the atretic follicles in both groups (Tables 4 and 5; Fig. 2A and B).

The cyclin D1 and E mRNA in small and large healthy follicles and cystic follicles was analyzed by RT-PCR. Both cyclins were detected in the samples (Fig. 4). Cyclin D1 mRNA were greater in the small and large follicles compared with cystic follicles. Cyclin E mRNA was elevated in small follicles with regard to cystic follicles, without differences with large follicles (Fig. 5).

Table 4
Immunohistochemical analysis of different proliferation and apoptotic proteins and DNA fragmentation in theca interna cells of cows from control and COD groups.

	Ki-67*	TUNEL*	CASP3**	BCL2**	BAX**	FAS**	FASLG**
C							
SF	10.83 ± 5.19^{b}	0.00 ± 0.00^{a}	3.83 ± 5.08^{a}	18.28 ± 17.12^{abc}	8.47 ± 5.45^{ab}	23.72 ± 15.32^{ac}	0.00 ± 0.00^{a}
TF	23.22 ± 9.40^{a}	0.00 ± 0.00^{a}	6.21 ± 3.40^{a}	28.30 ± 14.58^{c}	9.78 ± 3.03^{ab}	11.35 ± 6.76^{ab}	0.00 ± 0.00^{a}
AF	2.33 ± 0.82^{c}	3.75 ± 1.70^{b}	20.22 ± 9.23^{b}	6.52 ± 9.71^{ab}	32.30 ± 5.98^{c}	33.80 ± 5.97^{c}	2.60 ± 0.13^{b}
COD							
SF	13.67 ± 3.21^{b}	0.00 ± 0.00^{a}	5.34 ± 3.52^{a}	11.35 ± 9.15^{abc}	2.53 ± 2.05^{a}	10.94 ± 12.73^{ab}	0.00 ± 0.00^{a}
TF	12.75 ± 3.95^{b}	0.00 ± 0.00^{a}	6.88 ± 3.99^{a}	14.00 ± 5.41^{abc}	8.47 ± 2.45^{ab}	8.31 ± 4.78^{b}	0.00 ± 0.00^{a}
AF	3.25 ± 1.26^{c}	3.25 ± 1.25^{b}	27.14 ± 12.62^{b}	3.37 ± 2.78^{a}	33.44 ± 8.70^{c}	21.70 ± 11.26^{abc}	0.64 ± 0.77^{c}
CF	4.75 ± 1.71^{c}	0.00 ± 0.00^{a}	6.78 ± 4.77^{a}	22.47 ± 10.41^{bc}	15.82 ± 3.87^{b}	11.32 ± 6.26^{ab}	0.00 ± 0.00^{a}

The values represent mean ± standard deviation. Values in the same column with different superscripts (a–c) differ ($p < 0.05$). *Abbreviations*: C: control; COD: cystic ovarian disease; SF: secondary follicles; TF: tertiary follicles; AF: atretic follicles; CF: cystic follicles.
* Percentage of positive cells.
** IHCSA: immunohistochemical-stained area.

Table 5
Immunohistochemical analysis of different proliferation and apoptotic proteins and DNA fragmentation in theca externa cells of cows from control and COD groups.

	Ki-67*	TUNEL*	CASP3**	BCL2**	BAX**	FAS**	FASLG**
C							
SF	8.83 ± 5.00^{ab}	0.00 ± 0.00^{a}	–	12.96 ± 5.37^{a}	5.19 ± 2.45^{a}	14.99 ± 6.58^{ab}	–
TF	22.56 ± 11.61^{b}	0.00 ± 0.00^{a}	–	13.22 ± 3.72^{a}	3.15 ± 2.02^{a}	14.68 ± 12.11^{ab}	–
AF	5.00 ± 4.56^{a}	1.25 ± 0.50^{b}	–	3.99 ± 5.08a^{b}	21.40 ± 8.49^{b}	27.95 ± 10.84^{b}	–
COD							
SF	11.67 ± 3.00^{ab}	0.00 ± 0.00^{a}	–	3.56 ± 1.44^{b}	2.13 ± 1.34^{a}	18.88 ± 28.84^{ab}	–
TF	5.25 ± 2.06^{a}	0.00 ± 0.00^{a}	–	7.25 ± 2.32^{ab}	3.35 ± 1.57^{a}	3.09 ± 1.17^{a}	–
AF	4.25 ± 2.06^{a}	1.50 ± 0.57^{b}	–	2.62 ± 0.83^{b}	11.62 ± 7.38^{a}	16.07 ± 9.12^{ab}	–
CF	5.00 ± 2.45^{a}	0.00 ± 0.00^{a}	–	12.91 ± 7.14^{a}	8.01 ± 5.08^{a}	6.93 ± 4.98^{ab}	–

The values represent mean ± standard deviation. Values in the same column with different superscripts (a and b) differ ($p < 0.05$). *Abbreviations*: C: control; COD: cystic ovarian disease; SF: secondary follicles; TF: tertiary follicles; AF: atretic follicles; CF: cystic follicles.
* Percentage of positive cells.
** IHCSA: immunohistochemical-stained area.

3.4. Apoptosis evaluation

3.4.1. TUNEL

The greatest percentage of positive cells was found in granulosa cells of atretic follicles in both groups (Fig. 2E, Table 3). Few positive cells were detected in the categories remaining, including the cysts, consequently the quantification was impossible to be performed (Fig. 2D). Similar results occurred in relation to the theca cells (Tables 4 and 5).

3.4.2. Caspase-3

Immunostaining for caspase-3 was observed in the cellular nucleus and cytoplasm. In the granulosa, the most

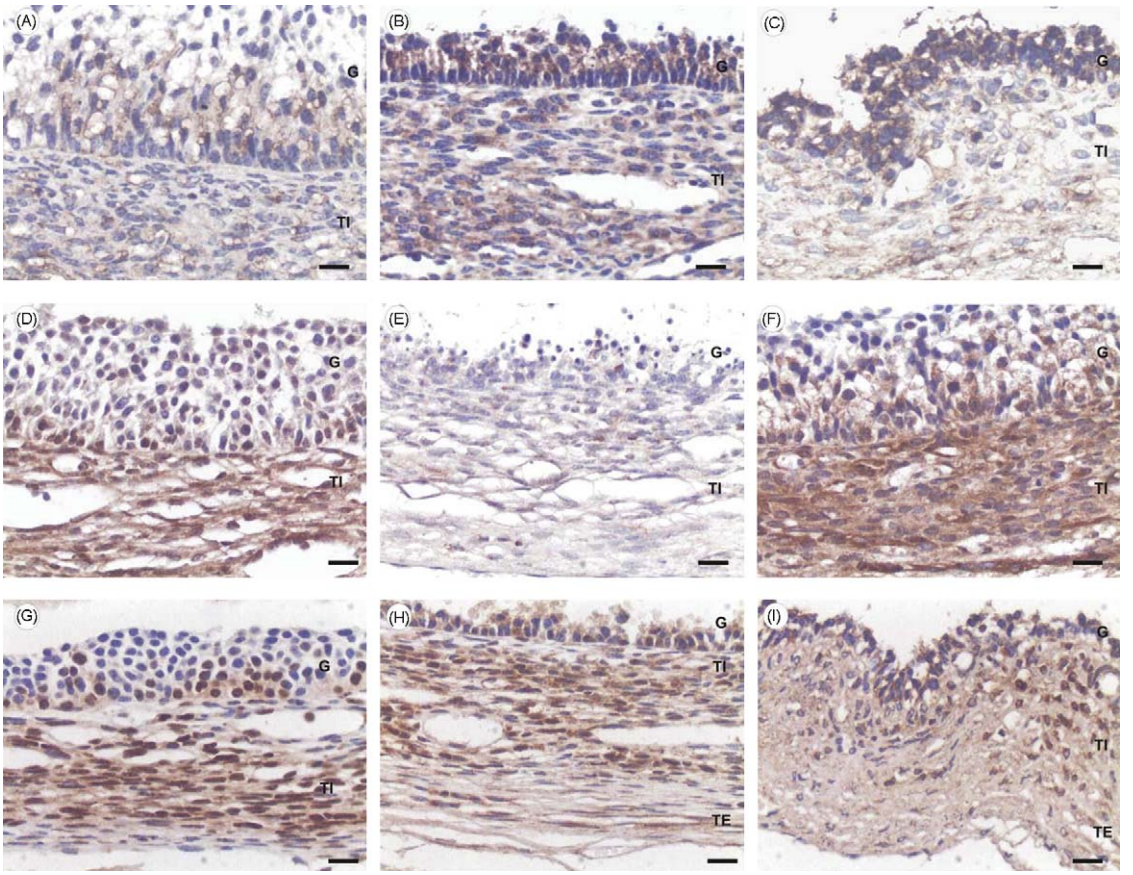

Fig. 3. Immunohistochemical localization of various BCL2 family proteins in healthy, atretic and cystic follicles of normal cycling and COD cows. Positive label is the brown staining of the cytoplasm of the cells. (A), (D) and (G) correspond to healthy tertiary follicles of control animals; (B), (E) and (H) correspond to atretic follicles of control animals and (C), (F) and (I) correspond to cystic follicles of cows with COD. (A–C) FAS immunolocalisation, (D–F) BCL2 immunolocalization and (G–I) BAX immunolocalization. Bars = 20 μm.

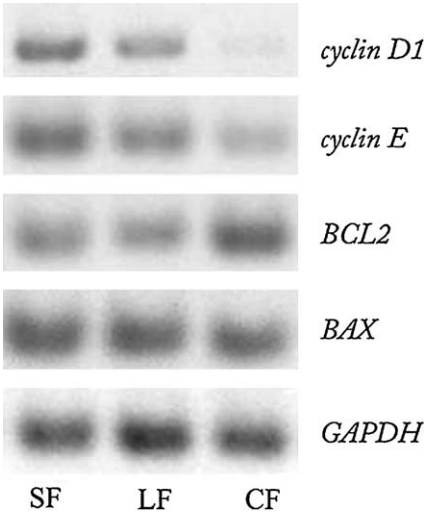

Fig. 4. Amounts of mRNA for cyclin D1, cyclin E, BCL2, BAX and GAPDH mRNA in ovarian tissues, as analyzed by RT-PCR. Lane 1: healthy small tertiary follicle (SF), lane 2: healthy large tertiary follicle (LF), lane 3: cystic follicle (CF).

intense staining was determined in the atretic follicles of both control and COD groups (Fig. 2H), and the least labelling was in the secondary follicles of the control group and tertiary follicles of both groups (Fig. 2G). Cystic follicles presented a similar pattern to tertiary follicles (Fig. 2I, Table 3). In the theca interna, a greatest relative amount was observed in the atretic follicles in both groups (Fig. 2H, Table 4). The weak immunostaining in the theca externa did not allow for quantification (Table 5).

3.4.3. FAS/FASLG components

In atretic follicles of both groups and cystic follicles, immunostaining for FAS was greater in granulosa cells than in healthy secondary and tertiary follicles (Fig. 3A–C, Table 3). In the theca interna, tertiary follicles presented less staining for FAS (Fig. 3A); whereas the theca externa showed a similar pattern to the theca interna but only tertiary follicles of the COD group showed minor immunostaining. FASLG immunoreactivity was present only in the granulosa and the theca interna cells of atretic follicles (Fig. 2F, Tables 4 and 5). There were no differences in immunostaining of FASLG between atretic follicles of the control and COD groups in granulosa cells. However, there was a greater staining in the theca cells of atretic follicles in the control group (Tables 4 and 5). FASLG was not detected in any follicular wall of healthy or cystic follicles and neither in the theca externa of all follicular categories.

3.4.4. BCL2 members

Using immunohistochemistry the relative protein expression of BAX and BCL2 in the components of the follicular wall was evaluated. The granulosa cells had greater immunostaining for BCL2 in the secondary and tertiary follicles in both groups and cystic follicles (Fig. 3D and F, Table 3) with a lesser intensity in atretic follicles (Fig. 3E,

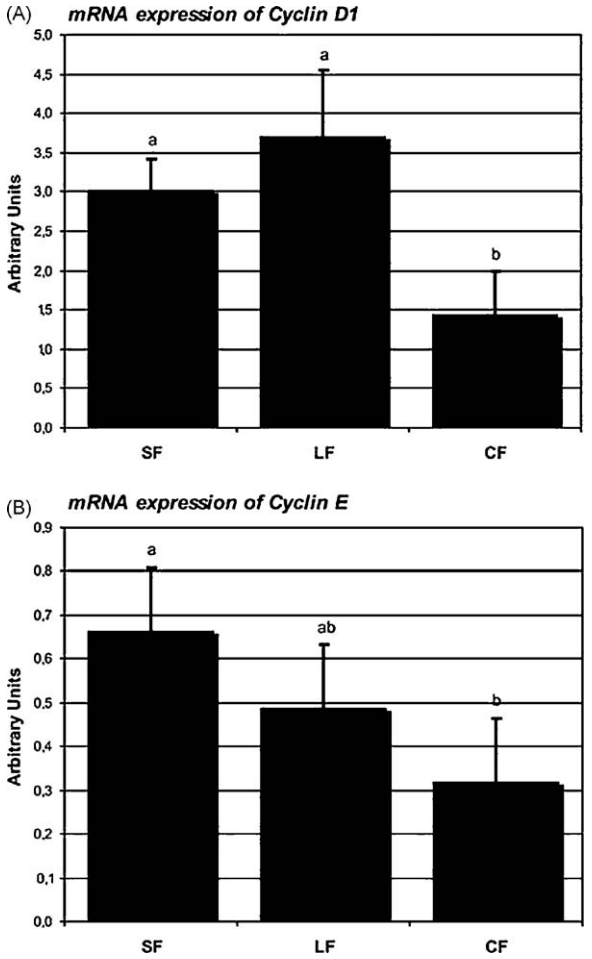

Fig. 5. (A and B) Semi-quantification of the mRNA of cyclin D1 and E in the wall of small (SF) and large (LF) healthy tertiary follicles and cystic follicles (CF). Bars with different superscript letters show significant differences. Values represent the mean ± SD.

Table 3). In the theca interna, BCL2 immunostaining was greater in tertiary follicles in relation to atretic follicles in both groups; cystic follicles showed differences with the atretic follicles from the same group (Fig. 3D–F). In the theca externa, little staining was observed in the atretic follicles of both groups as well as in the secondary follicles of the COD group. By contrast, immunostaing for BAX in granulosa and theca cell layers was greater in the atretic follicles of both groups (Fig. 3H, Tables 3 and 4). The values for cystic follicles were similar to those in the tertiary follicles of controls (Fig. 3G and I, Table 3). The immunostaining in theca externa of atretic follicles of COD animals was not different from the other follicular categories.

The relative mRNA of BAX and BCL2 was analyzed by RT-PCR (Fig. 4). The integrity of mRNA was preliminarily assayed by *GAPDH* RT-PCR analysis (Fig. 4). The BCL2 and BAX was assessed in healthy small and large follicles of control ovaries and cystic follicles. The greater amount of BCL2 mRNA (201 bp) was evident in cystic follicles related to small and large follicles in all samples analyzed (Fig. 6). The BAX mRNA (431 bp) was detected in all samples without

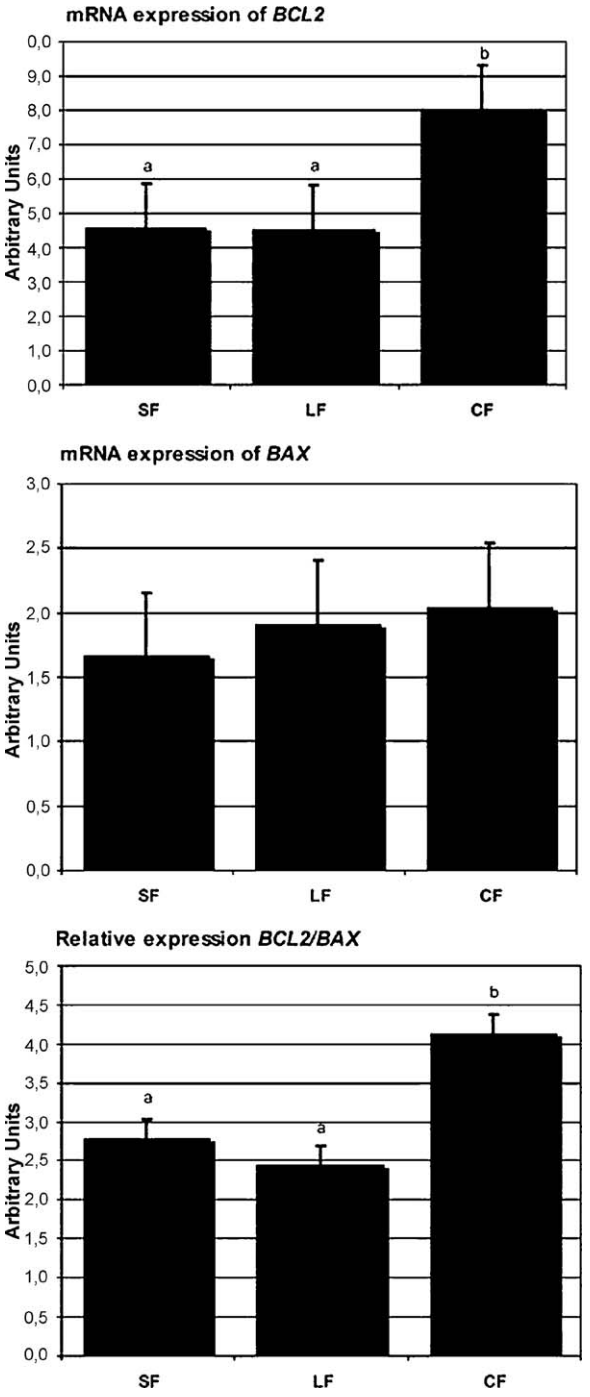

Fig. 6. Semi-quantification of the mRNA of BCL2, BAX and the BCL2/BAX ratio in the wall of small (SF) and large (LF) healthy tertiary follicles and cystic follicles (CF). Bars with different superscript letters show significant differences. Values represent the mean ± SD.

differences between groups (Fig. 6). Multiplex PCR analysis was also used to compare the relative amounts of transcriptional activity for BCL2 and BAX in the samples and indicated a predominance of BCL2 as compared with BAX gene expression in cystic follicles (Fig. 6).

4. Discussion

In the present study, there were significant alterations in cell proliferation and apoptosis rate in follicles from cows with induced ovarian cysts. These findings support the notion that follicular persistence is an important component of COD pathogenesis.

The results in the present study indicate a clear decrease in the index of proliferation in all layers of cystic follicles in animals with COD, similar to that observed in the atretic follicles. Similar to what was observed in samples of the follicular wall, amounts of mRNA for D1 and E cyclins decreased in the cystic follicles compared with healthy tertiary follicles. In agreement with the present findings, Isobe and Yoshimura (2000a,b) detected a lesser index of proliferation in all follicular layers of ovarian cysts in cattle. In the previous research, an intense proliferation in the basal area of the granulosa layer of normal tertiary follicles, and decrease in atretic and cystic follicles was observed. These results are consistent with those found in induced cystic follicles in rats in different experimental models (Baravalle et al., 2006, 2007; Salvetti et al., 2009).

In the ovary, estradiol, FSH and LH are essential signals for the growth of preovulatory follicles and their subsequent terminal differentiation as CL. Each hormone acts via specific receptors and intracellular signalling pathways. The LH surge inverts the balance of positive versus negative cell cycle regulators and triggers granulosa cell exit from the cell cycle concurrent with luteinisation (Robker and Richards, 1998b). Previous research suggests one putative mechanism by which the LH surge terminates granulosa cell proliferation through rapid inhibition of cyclin D2 transcription. This is what usually happens in each estrous cycle in cattle; however, a variety of hormonal changes that influence the ovarian homeostasis occurs in COD. Ribadu et al. (2000) showed that LH pulse frequency (but not features of FSH secretion) was reduced during cyst formation and persistence in the ACTH induction model, which suggests that alterations in the pulsatile release of LH might cause ovarian follicular cyst formation in heifers. Changes in the normal pattern of LH secretion affect the presence of LH receptors as well as steroid hormone receptors. LH receptor mRNA is increased in the follicles of cows with COD (Calder et al., 2001), whereas estrogen receptor 2 is diminished in follicles of cows with COD (Salvetti et al., 2007). Several growth factors such as insulin-like growth factor-1 (IGF1) also promote granulosa cell survival (Johnson, 2003). In ovarian cysts, an altered amounts of IGF1 protein was observed that could be related to reduced cell proliferation in these structures (Ortega et al., 2008; Rey et al., 2009). This information from previous and the present study indicate these hormonal changes result in altered amounts of proteins involved in the cellular cycle such as cyclins D1 and E, which contribute to follicular persistence.

By contrast, DNA fragmentation, caspase-3, FASLG and BAX protein were significantly less in layers of tertiary and cystic follicles from COD cows than in normal atretic follicles of both groups, whereas BCL2 was greater in growing and cystic follicles in both groups. No differences were found in FAS in atretic and cystic follicles, being greater than in secondary and tertiary follicles. The BAX and BCL2

proteins were coincident with the gene expression evaluations in the present study being similar in tertiary and cystic follicles. Moreover, there was a relatively greater BCL2 than BAX in cystic follicles as compared to tertiary follicles, as indicated by multiplex analysis. Previous results evaluated apoptosis *in situ* and associated proteins in ovarian cysts of cattle (Isobe and Yoshimura, 2000b, 2007), as well as in different experimental models of cystic ovaries induction in rats (Anderson and Lee, 1997; Shirwalkar et al., 2007; Salvetti et al., 2009). In studies conducted in women with polycystic ovarian syndrome, Das et al. (2008) found greater gene expression of anti-apoptotic factors and greater amounts of proteins for factors such as cellular inhibitor of apoptosis proteins and BclxL and less BAX and caspase-3 in granulosa cells of cystic follicles, with similar values to those found in healthy antral follicles.

Tropic hormones important for cell proliferation also have a role in the suppression of apoptosis or maintenance of cell survival. FSH and LH are important factors involved in the proliferation of follicular somatic cells and development of preovulatory follicles. Hypophysectomy leads to massive apoptosis of developing follicles by the suppression of serum gonadotropins, whereas the treatment of cultured follicles with gonadotropins prevents the spontaneous onset of follicular apoptosis in serum-free cultures, suggesting that gonadotropin functions as a follicle survival factor (Chun et al., 1994). Apoptosis occurs by default when cells fail to receive the signals required to suppress the death program. Signals for the induction of apoptosis can also be mediated by apoptotic factors such as TNF and FASLG (Hsu and Hsueh, 1997).

In cattle, during the first follicular wave of the estrous cycle, expression of FAS and FASLG genes is elevated in subordinate follicles compared with dominant follicles (Porter et al., 2000, 2001). *In vitro* studies (Quirk et al., 2004) indicate granulosa cells possess endogenous pathways to activate apoptosis that are inhibited in the presence of survival factors. For example, granulosa cells of cattle express the FAS gene but are resistant to death by exogenous FASLG *in vitro* when serum is present in the culture medium (Porter et al., 2000; Vickers et al., 2000; Quirk et al., 2000, 2004). In the present study, the FAS gene is expressed similarly in atretic and cystic follicles but the FASLG gene is expressed only in atretic follicles, which could mean the apoptotic machinery has not been activated because of the absence of the ligand. The increased availability of survival factors such as IGF in the dominant follicles of cattle *in vivo* inhibits the expression of the FAS and FASLG genes and prevents activation of the FAS pathway. The effect of growth factors such as IGF1 to protect cells from apoptosis seems to be correlated with their ability to stimulate progression through the cell cycle. IGF1, basic fibroblast growth factor and epidermal growth factor all decreased the FASLG-induced apoptosis of cultured granulosa cells of cattle and simultaneously increased cell proliferation (Quirk et al., 2004). Follicular cysts have greater amounts of IGF2, a growth factor that acts to prevent cellular death (Rey et al., 2009).

By contrast, the inhibition of granulosa cell apoptosis and follicular atresia mediated by gonadotropin treatment might be linked to the ability of gonadotropins to reduce the amount of BAX present in granulosa cells while maintaining a constitutive amount of anti-apoptotic factors such as BCL2 and BclxL (Tilly, 1996). Furthermore, gonadotropin treatment might contribute to the shift in the balance of death inducer to death repressor gene expression. It is also possible that other hormonal signalling such as ovarian steroids or locally produced growth factors that can influence granulosa cell fate and serve as the primary regulators of BCL2 and BCL2L1 gene expression (Tilly et al., 1991; Johnson, 2003;). With regard to the pro-apoptotic protein BAX, Bax-deficient mice have abnormal follicles with an excessive number of granulosa cells (Knudson et al., 1995).

Both pathways of apoptosis – exogenous through death receptors or endogenous by the BCL2 gene family – seem to retard apoptosis in follicular cysts of cattle. The differences in the hormonal concentrations characteristic of COD that affect the cellular proliferation might also alter the balance of pro-/anti-apoptotic proteins as well as the FAS/FASLG components, leading to reduced caspase-3 and a scarce *in situ* apoptosis that contributes to the persistence of the cysts.

5. Conclusion

In summary, findings in the present study indicate cellular proliferation and apoptosis are altered in the cystic follicles of cattle. The present study provides new insights into the molecular mechanisms underlying the aberrant persistence of follicular cysts. The hormonal change characteristics of the disease might influence the amount of proteins related to the cellular cycle and death processes thereby contributing to follicular persistence. Future studies could further expose the complexities of COD in cattle and help develop novel preventive or therapeutic strategies.

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