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Characterization of Cytoskeletal Proteins in Follicular Structures of Cows with Cystic Ovarian Disease

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Summary

The distribution of intermediate filaments (vimentin, cytokeratins, desmin) and microfilaments (α-smooth muscle actin and muscle specific actin) was studied immunohistochemically in bovine ovaries, with and without cystic ovarian disease. The immunohistochemically stained area (IHCSA), was quantified by image analysis, to evaluate the expression of these cytoskeletal proteins in the follicular wall of healthy antral, atretic, and cystic follicles. The granulosa cell layer of cystic follicles and atretic follicles had a significantly larger IHCSA for vimentin than did healthy antral follicles. Cytokeratins reacted lightly in the granulosa cells of antral follicles of normal ovaries, whereas granulosa cells of atretic and cystic follicles showed significantly higher IHCSA values. Immunohistochemical localization of desmin, muscle specific actin, and α-smooth muscle actin was restricted to the theca externa. This study supports earlier suggestions that strongly positive reactions with vimentin and cytokeratin antibodies observed in the granulosa cells of cystic follicles are due to the reorganization that occurs in the follicle during the process of cystic development, and are associated with changes in the expression of cytoskeletal proteins that are essential to proper cellular functioning.

Keywords: cattle; cystic ovary; follicular cysts; ovarian disease

Introduction

Cystic ovarian disease (COD) is one of the most common causes of reproductive failure in cattle (Peter, 2004). The incidence of COD in dairy cattle has been reported to range from 5.6% to 18.8% (Peter, 2004; Silvia *et al.*, 2005). This estimate may be too low, as more than 60% of cows that develop COD before the first post-partum ovulation recover spontaneously and remain undetected (Peter, 2004). A cystic follicle, in cattle, has been defined as an anovulatory follicle-like structure (more than 20 mm in diameter) that may persist in the ovary (usually for more than 10 days), with or without a corpus luteum (CL) (Silvia *et al.*, 2002; Peter, 2004).

The mechanisms that lead to the development of follicular cysts have been the object of speculation and research for many years (Wiltbank *et al.*, 2003), but are still poorly understood. It is believed that the disease has a multifactorial aetiology (Peter, 2004). Follicular cysts appear to be caused by an endocrine imbalance in the hypothalamo-hypophyseal-gonadal axis (Lopez-Diaz and Bosu, 1992; Hamilton *et al.*, 1995). Abnormal production of luteinizing hormone (LH) was reported in cows with cystic follicles by Hamilton *et al.* (1995). Since LH largely affects the function of the granulosa and theca interna layers (Voss and Fortune, 1993), altered steroidogenesis may occur in the cystic follicles (Isobe *et al.*, 2003). By contrast, although many studies have outlined the dynamics of follicular growth, our understanding of the cellular and molecular

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changes that occur within the ovarian follicle leading to anovulation is still incomplete. Cellular changes may be in the form of an aberrant production of growth factors by the granulosa cells, inappropriate secretion of extracellular matrix (ECM) proteins, or changes in the cytoskeletal proteins (Peter *et al.*, 1995; Salvetti *et al.*, 2003, 2004).

The cytoskeleton is composed of three types of high molecular weight polymers, namely microtubules, microfilaments, and intermediate filaments. Microtubules and microfilaments are homogeneous and ubiquitous structures (Feuilloley and Vaudry, 1996), whereas intermediate filaments represent a heterogeneous family of fibres whose expression depends upon the level of differentiation of the cells (Goldman *et al.*, 1990). In exocrine (Sinha and Wagner, 1987) and endocrine cells (Ravindra and Grosvenor, 1990), which secrete their products by exocytosis, microtubules and microfilaments play a role in both the traffic of secretory granules in the cytoplasm and the fusion of the vesicles with the plasma membrane. Although the presence of vesicles has been observed in steroidogenic cells, it is generally accepted that steroids are released immediately after synthesis, by diffusion through the plasma membrane (Bomsel *et al.*, 1986). However, during the last decade, a number of studies have demonstrated that pharmacological agents that induce either disruption or stabilization of cytoskeletal fibres strongly affect steroid hormone secretion (Chen *et al.*, 1994). This factor is particularly important in the changes in steroidogenesis that may take place during cystogenesis (Isobe *et al.*, 2003).

Cytoskeletal proteins have been extensively examined in the ovarian cells of many species, including laboratory animals, farm animals, and human beings (Selstam *et al.*, 1993; van den Hurk *et al.*, 1995; Khan-Dawood *et al.*, 1996; Löffler *et al.*, 2000; Marettova and Maretta, 2002). However, the changes that occur in pathological situations, such as the development of ovarian cysts, have been studied only in experimental models. Thus, in induced follicular cysts in rats, structural and functional changes occur during cystogenesis, and these may be associated with changes in the expression of cytoskeletal proteins (Salvetti *et al.*, 2004).

It has been hypothesized that cytoskeletal proteins contribute to the structural integrity of cells (Schliwa and Van Blerkom, 1981) and participate in cell-to-cell binding and in differentiation events (Luna and Hitt, 1992). The purpose of this study was to evaluate the role of intermediate filament and microfilament proteins during cystogenesis in cows, by examining the pattern of expression in normal and cystic bovine ovaries.

Materials and Methods

Collection and Preparation of Tissues

Ovaries with ($n = 12$) or without ($n = 12$) cystic follicles were collected at a local abattoir in the city of Rafaela (Santa Fe, Argentina), within 20 min of death, from mixed breeds of cows, assessed visually as non-pregnant.

Macroscopical abnormalities, other than ovarian cysts, were not observed in the reproductive tract of any of the cows. Follicular cysts were diagnosed when the follicles were more than 20 mm in diameter, in the absence of a functional CL in either right or left ovary (Brown *et al.*, 1982). The cystic follicles used in this study showed no signs of luteinization.

Ovaries without cystic follicles were used for the observation of antral and atretic follicles. The stage of the oestrous cycle was defined by macroscopical observation of the ovaries (colour, consistency, CL stage, number and size of follicles) and the uterus (colour, consistency and mucus) (Berisha *et al.*, 2004).

The ovaries were dissected, sectioned, and fixed in 10% buffered formalin for 6 h at 4 °C and washed in phosphate-buffered saline (PBS). For light microscopy, fixed tissues were dehydrated in an ascending series of ethanol concentrations, cleared in xylene, and embedded in paraffin wax. Serial sections (5 μm), were mounted on 3-aminopropyl triethoxysilane (Sigma, St Louis, MO, USA)-coated slides and dried for 24 h at 37 °C (Ortega *et al.*, 2004).

Classification of Follicles

Follicles were classified macroscopically and microscopically according to the criteria listed in the *Nomina Histologica* (1994). Only follicles that appeared healthy (i.e., well vascularized and with a transparent follicular wall and fluid) and whose diameter was >5 mm were used and classified as antral. The selection of this follicle-size category was based on the reported gonadotropin dependence and changes in the expression of steroidogenic enzymes and LH receptor mRNAs. Xu *et al.* (1995) showed that at this size follicle growth will be halted if follicle-stimulating hormone (FSH) is suppressed. The atretic follicles were sub-classified as obliterative or cystic, based on the descriptions in Table 1.

Cystic follicles were initially classified microscopically into types 1, 2 or 3, on the basis of their granulosa cell layer structure (Isobe and Yoshimura, 2000); however, they were later considered to be a single group, due to lack of differences between the types in respect of the parameters studied (preliminary data not published). At least 20 follicles of each type were examined, except in the case of cystic follicles, of which 12 were analysed.

Table 1
Classification of the atretic follicles according to previous classifications of atresia

Present study	*Irving-Rodgers* et al. *(2001)*	*Marion* et al. *(1968)*	*Rajakoski (1960)*
Atretic obliterative	Antral atresia	Definite atresia (contracting or obliterative)	Obliterative atresia (first, second and third degree)
	Basal atresia	Late atresia (previously large follicle)	
Atretic cystic	Advanced antral atresia	Definite atresia (cystic)	Cystic atresia

Immunohistochemistry

Details and concentration of antibodies used are summarized in Table 2. Each antibody was assayed in at least five sections of each ovary from each animal. A streptavidin-biotin immunoperoxidase method was used as previously described (Ortega *et al.*, 2004; Salvetti *et al.*, 2004). In brief, sections were dewaxed, hydrated, and then subjected to microwave pre-treatment for antigen retrieval. Endogenous peroxidase activity was inhibited with 1% H_2O_2 and non-specific binding was blocked with 10% normal goat serum. All sections were incubated with primary antibodies for 18 h at 4 °C. The samples were washed and then incubated for 30 min at room temperature with pre-absorbed biotinylated secondary antibodies, selected for each of the two types of primary antibody used (monoclonal or polyclonal). Antigens were detected 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 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 by rabbit and mouse non-immune serum (Dako, Carpinteria, CA, USA). The specificity of the secondary antibodies was tested by incubation with primary antibodies of proven negative reaction with bovine antigen (anti-CD45 [Clone PD7/26 and 2B11; Dako] and anti-Ki-67 [polyclonal, rabbit anti-human Ki-67, Dako]). Sections of small intestine and skin were used as positive controls.

Image Analysis

Image analysis of the immunoreaction in tissue sections was performed by colour segmentation analysis with the Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver Spring, MA, USA) (Ortega *et al.*, 2004; Salvetti *et al.*, 2004). Briefly, images were digitized with a CCD-colour video camera (Sony, Montvale, NJ, USA) mounted on a conventional light microscope (Olympus BH-2, Olympus Co., Tokyo, Japan), with an objective magnification of × 40. The details of image analysis as a valid method for quantifying expression, and the methodological details, were described previously (Ortega *et al.*, 2004). The microscope was prepared for Koehler illumination by recording a reference image of an empty field for the correction of unequal illumination (shading correction) and by calibrating the measurement system with a reference slide to determine background threshold values. The reference slides contained a series of tissue sections processed in the absence of a primary antibody. The immunoreactivity in the wall of the blood vessels was used to provide internal controls, and positive controls were used as interassay controls to maximize the level of accuracy of the method (Ranefall *et al.*, 1998).

Microscopical fields covering the entire follicular wall area were digitized and stored in a 24-bit true colour tagged image file format (TIFF). The resolution of the images was set to 640 × 480 pixels. At this magnification, each pixel of the image corresponded to 0.26 μm, and each field represented a tissue area of 0.02 mm^2.

To obtain quantitative data regarding immunohistochemical reactivity in the follicular wall, at least three sections for each specimen and antibody were evaluated and 50 representative fields were analysed. By means of AutoPro macro language, an automated sequence operation was created to measure the immunohistochemically stained (labelled) area (IHCSA). The IHCSA was calculated as a percentage of total area evaluated by colour segmentation analysis, which extracts objects by locating all objects of a specific colour (brown label). The brown label was selected and a mask was then applied to make the separation of colours permanent. The images were then transformed to a bi-level scale TIFF. The IHCSA (black area) was calculated from at least 50 images of each area (granulosa, theca externa, and theca interna) in each slide (Salvetti *et al.*, 2004).

The data were expressed as the mean ± standard error of mean (SEM). The IHCSA of each antibody in different areas and structures and the morphometric data were analysed by means of the ANOVA test and the Duncan post-test.

Table 2
Antibodies, suppliers and dilutions

Antibodies	*Clone*	*Supplier*	*Dilution*
Primary antibodies			
α-Smooth muscle actin (α-SMA)	αsm-1	Novocastra	1 in 50
Muscle specific actin	HHF35	Biogenex	Prediluted
Vimentin	V9	Zymed	Prediluted
Cytokeratins (1/5/6/8/10/14/18)	LP 34, 34βE12 & 35βH11	Dako	Prediluted
Desmin	ZC18	Zymed	Prediluted
Secondary antibodies			
Anti-rabbit IgG	Polyclonal	Zymed	1 in 300
Anti-mouse IgG	Polyclonal	Chemicon	1 in 120

Results

Ovarian Morphology

Ovaries from normal cows exhibited follicles in various stages of development, including primary, secondary and tertiary follicles, corpora albicans and CL, as well as follicles with different degrees of atresia. In cows with COD, small follicles in early development could be observed, in addition to others showing evidence of atresia, and one or two large cystic structures with a thickened or absent granulosa cell layer. A CL was absent in all COD cases.

Immunohistochemistry

A summary of the immunohistochemical expression of the different antibodies is given in Table 3. The reactions were negative in all negative controls and positive in the positive tissue controls. Smooth muscle cells positive for α-SMA were present in the cortical and medullary regions. Some small scattered clusters of cortical spindle elements beneath the tunica albuginea showed the presence of α-SMA. Perifollicular structures (resembling smooth muscle cells) with positive reactivity for muscle specific actin and α-SMA were incorporated in the theca externa, forming an incomplete sphere around antral, atretic and cystic follicles. The granulosa and theca interna layers were negative for this antibody. In the medulla, α-SMA and muscle specific actin-positive cells were concentrated in the walls of blood vessels. Significant differences in IHCSA between groups were not observed (Fig. 1).

Desmin was present in stromal cells occupying the medullary and cortical regions. In the cortical zone, desmin-positive structures were restricted to a small number of positive cells in the theca externa around antral, atretic and cystic follicles; however, significant differences were not observed. In addition, desmin-positive cells occasionally formed clusters and circular accumulations representing the remnants of blood vessels in atretic follicles. Except in these areas, the positive reaction was randomly distributed. In the medullary zone, desmin positivity was mainly localized in the smooth muscle cells of the tunica media layer of blood vessels. The granulosa and theca interna layer was negative (Fig. 1).

Vimentin immunoreactivity was consistently observed in the granulosa cell layer of healthy, atretic and cystic follicles. This protein had a significantly larger IHCSA in the granulosa cell layer of atretic and cystic follicles than in antral follicles. The theca interna cells showed moderate immunoreactivity without significant differences in the IHCSA. A significant increase in vimentin IHCSA was observed in the theca externa of cystic follicles and atretic cystic follicles. In addition, the endothelial-cell lining of blood capillaries in stroma, atretic follicles and larger blood vessels was strongly positive for vimentin (Fig. 2).

There was low expression of cytokeratins in the granulosa cells of antral follicles in normal ovaries, while in granulosa cells of atretic and cystic follicles the IHCSA was significantly higher. Low immunoreactivity was detected in the theca layers (Fig. 3).

Discussion

The study demonstrated that cystogenesis in cows is associated with simultaneous changes in the expression of cytoskeletal proteins that play a role in the formation of cell contacts and in determining cell shape. Several characteristics of the alterations observed in the intermediate filaments in cystic ovaries were also observed during atresia in normal ovaries (Khan-Dawood *et al.*, 1996; Marettova and Maretta, 2002). These previous studies showed that the processes of follicular atresia and the formation of the CL in the ovary are also associated with dramatic changes in the organization of the cytoskeleton in the cells that are undergoing atresia and luteinization.

Some differences were observed between cystic and obliterative atretic follicles, possibly related to the different pathways of the atresia processes, for example,

Table 3
Distribution of immunoreactive cytoskeleton proteins in the follicular wall of antral, atretic and cystic follicles

Cytoskeletal protein and site in follicular wall	*Mean IHCSA ± SEM in follicles of the stated type*			
	*Antral**	*Atretic obliterative**	*Atretic Cystic**	*Cysts*
Desmin				
Granulosa layer	—	—	—	—
Theca interna	—	—	—	—
Theca externa	11.32 ± 1.81	9.94 ± 1.08	12.46 ± 1.76	8.57 ± 1.59
Vimentin				
Granulosa layer	4.8 ± 1.24 [a]	34.71 ± 2.32 [b]	27.88 ± 6.25 [b]	32.67 ± 2.74 [b]
Theca interna	4.66 ± 1.13	6.21 ± 1.05	5.6 ± 0.92	4.92 ± 1.72
Theca externa	2.81 ± 1.04 [a]	3.9 ± 0.59 [a]	7.67 ± 1.5 [b]	10.53 ± 079[b]
Cytokeratins				
Granulosa layer	8.68 ± 0.9 [a]	25.27 ± 3.53 [b]	29.05 ± 4.73 [b]	20.94 ± 2.89 [b]
Theca interna	2.74 ± 0.59	3.08 ± 0.83	2.20 ± 0.17	2.62 ± 1.6
Theca externa	0.44 ± 0.15	0.27 ± 0.12	0.50 ± 0.32	0.62 ± 0.21
Muscle Specific Actin				
Granulosa layer	—	—	—	—
Theca interna	—	—	—	—
Theca externa	15.76 ± 1.91	18.56 ± 3.06	16.33 ± 1.54	23.31 ± 3.23
α-Smooth Muscle Actin				
Granulosa layer	—	—	—	—
Theca interna	—	—	—	—
Theca externa	14.09 ± 2.38	12.97 ± 2.22	15.69 ± 3.0	16.45 ± 2.05

*Controls.
IHCSA: Immunohistochemical stained (labelled) area. In horizontal rows, entries with different superscript letters differ significantly ($P < 0{,}05$).

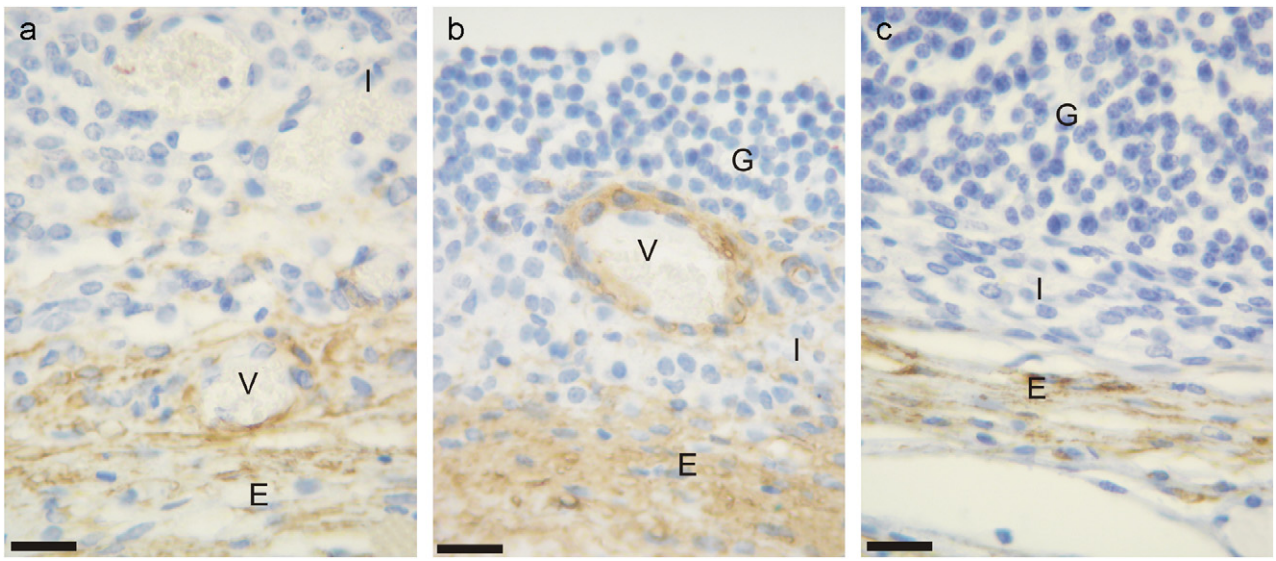

Fig. 1a–c. Immunohistochemical reaction of antral follicles with antibodies against (a), muscle specific actin, (b) α-smooth muscle actin, and (c) desmin. Immunolabelling is restricted to the theca externa (E) and blood vessels (V). The granulosa layer (G) and theca interna (I) are negative. Bars, 20 μm.

the invasion of the follicular wall by other cellular types (Irving-Rodgers *et al.*, 2001, 2004). Contrary to findings in human beings (Czernobilsky *et al.*, 1985), in bovine ovaries only part of the granulosa cell population in antral follicles was positive to vimentin antibody. Homogeneous reactivity was observed in the granulosa cells of atretic follicles, thus according with earlier findings (van den Hurk *et al.*, 1995). In cystic

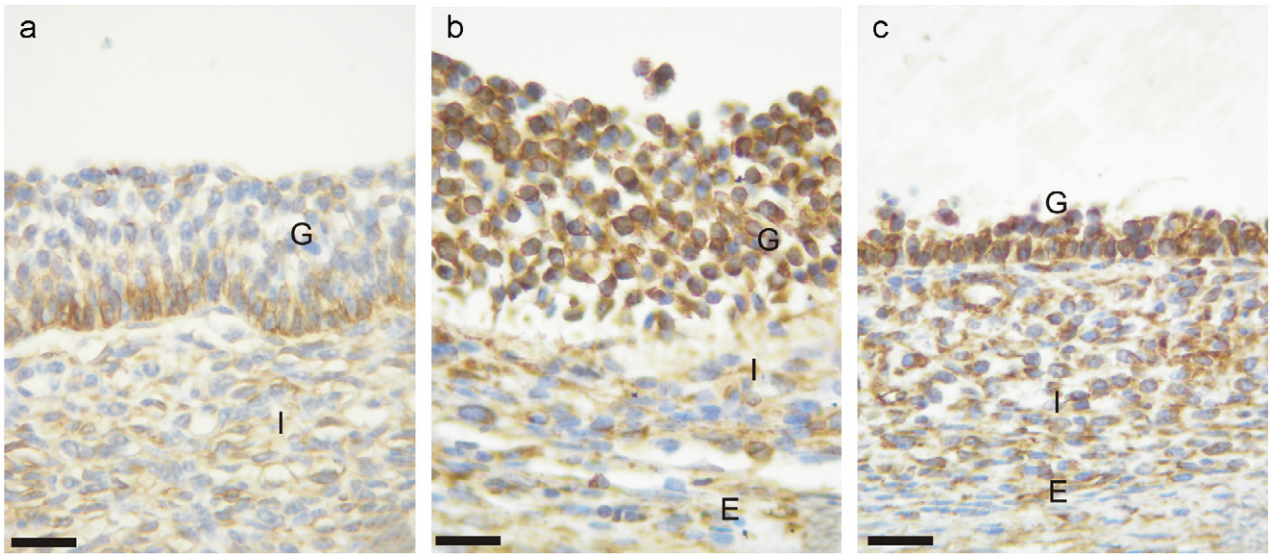

Fig. 2a–c. Immunohistochemical reaction with vimentin antibody in (a) antral, (b) atretic obliterative, and (c) cystic follicles. The reactivity is intense in the granulosa (G) and theca externa (E) of atretic and cystic follicles. The theca interna (I) shows a moderate positive reaction. Bars, 20 µm.

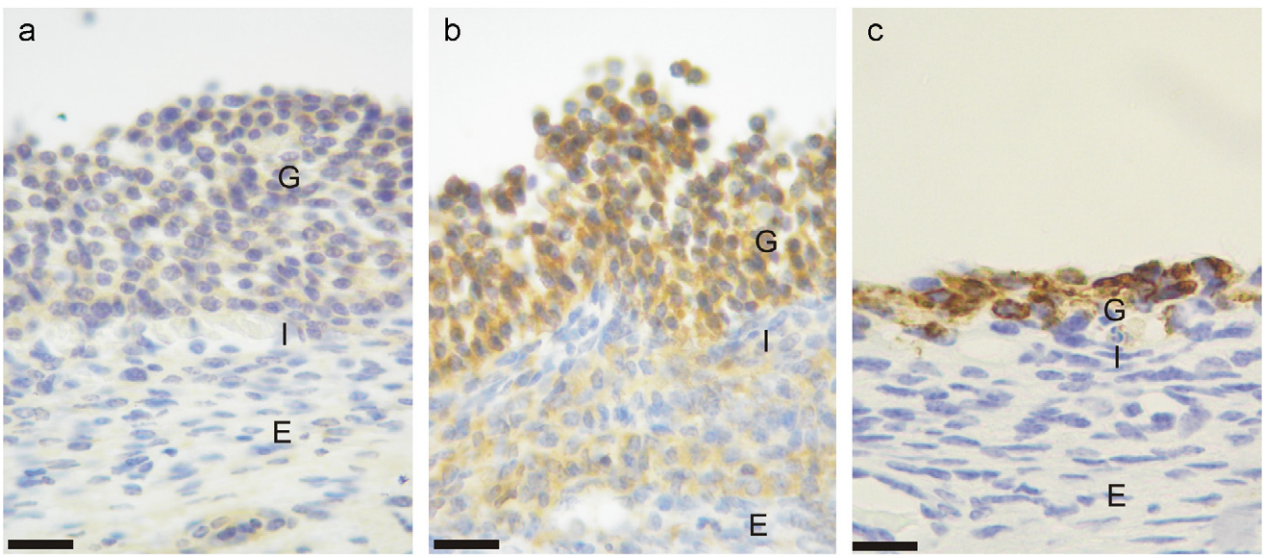

Fig. 3a–c. Immunohistochemical reaction for cytokeratins in (a) antral, (b) atretic obliterative, and (c) cystic follicles. The granulosa layer (G) of atretic and cystic follicles is strongly positive. The theca interna (I) and theca externa (E) are weakly positive. Bars, 20 µm.

follicles, the reactivity was similar to that in atretic follicles, as in our previous results in experimental models of COD in rats (Salvetti *et al.*, 2004).

This study showed that the changes in the organization of cytoskeletal elements in the follicular wall of cystic follicles were followed by changes in the synthesis of the cytoskeletal proteins (vimentin and cytokeratins). The fact that the follicular cells produce relatively large amounts of these proteins could be interpreted as (1) a sign of stimulation of cytoskeletal protein synthesis by cells developing stress fibres, or (2) a "down-regulation" during cell growth or proliferation (Ben-Ze'ev and Amsterdam, 1987). The occurrence of vimentin-containing granulosa cells has been related to mitotic activity, follicular atresia and de-differentiation associated with loss of cell-to-cell contact (van den Hurk *et al.*, 1995). However, if cell proliferation in the granulosa and theca interna layers decreases in association with the development of follicular cysts (Isobe and Yoshimura, 2000), the changes in vimentin expression observed would be due to cellular de-differentiation.

On the other hand, the expression of cytokeratins was significantly higher in granulosa cells of atretic and cystic follicles than in antral follicles; this accords with previous findings in rats (Salvetti *et al.*, 2004). In human ovaries, Czernobilsky *et al.* (1985) showed that the surface epithelial cells were positive to antibodies against cytokeratins and vimentin. Granulosa cells of follicles in all stages were also positive for cytokeratins and vimentin. As the follicle matured, the cytokeratin content usually appeared to decrease, whereas vimentin remained unchanged (Khan-Dawood *et al.*, 1996; Löffler *et al.*, 2000).

Anderson and Lee (1997) showed that in a rat COD model induced by dehydroepiandrosterone, the basal layer of granulosa cells lost their vimentin filaments and acquired keratin. They concluded that the basal layer of granulosa cells was transformed into an epithelium during cystogenesis and that this was an example of the mesenchymal-epithelial transformation associated with the pathological process.

The notion that an increase in intermediate filaments represents a degenerative change is supported by several studies (Ghadially *et al.*, 1978; Pieraggi *et al.*, 1984). It is possible that modifications occurring during the development of ovarian cysts in cattle are related to those that take place during the atresia process and include degenerative changes. Ovarian follicular atresia is reported to be associated with degeneration of the granulosa layer (Hirshfield and Midgley, 1978), fragmentation of the basal lamina (Bagavandoss *et al.*, 1983), reduced DNA synthesis (Hirshfield and Midgley, 1978; Greenwald, 1989), decreased oestrogen production (Maxon *et al.*, 1985) and decreased gonadotrophin binding (Maxon *et al.*, 1985). It is also associated with the development of apoptosis in the follicles (Amsterdam *et al.*, 2003; Johnson, 2003) and cytokeratin and vimentin expression.

No differences were observed in the expression of desmin, muscle-specific actin or α-SMA, suggesting that their expression is related to stable structures that either do not participate or do not undergo changes during the atresia or cystogenesis. Their expression pattern, limited principally to the theca externa and stroma, was in keeping with that described in previous reports (van den Hurk *et al.*, 1995; Marettova and Maretta, 2002).

In conclusion, this study supports earlier observations (Salvetti *et al.*, 2004) suggesting that the high intensity of binding with anti-vimentin and anti-cytokeratin antibodies observed in the granulosa cells of the cystic follicles is probably due to structural and functional changes that occur during cystogenesis. It is possible that these changes are associated with modifications in the expression of cytoskeletal proteins that may be necessary for correct cellular functioning.

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