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HISTOCHEMICAL STUDY OF THE EXTRACELLULAR MATRIX COMPONENTS IN THE FOLLICULAR WALL OF INDUCED POLYCYSTIC OVARIES

Natalia R. Salvetti [1,2], Eduardo J. Gimeno[4], Ana M. Canal[3], Juan A. Lorente[1] and Hugo H. Ortega[1,2]

[1] Department of Histology and Embryology, [2]Center for Experimental Biology and Laboratory Animal Sciences, [3]Department of Basic Pathology, Faculty of Veterinary Sciences, National University of Litoral (UNL),[4]Institute of Pathology, Faculty of Veterinary Sciences, National University of La Plata (UNLP), Argentina.

ABSTRACT

Numerous models have been developed to study polycystic ovarian syndrome in rats. In the present study, the syndrome was induced by exposure to constant light. The histological structure and differential distribution of extracellular matrix (ECM) fibers as well as the glycosaminoglycans (GAGs) content and composition of the ovarian follicular wall of rats with polycystic syndrome were evaluated. Histochemical differences were observed in the granulosa and theca externa of follicular cysts when compared to normal preovulatory follicles. The collagen content of the theca externa of follicular cysts, quantified by the picrosirius method, was higher than in the controls. The neutral carbohydrate and acidic GAG levels were lower in the granulosa and higher in the theca externa of cyst follicles than in control ovaries. Histomorphometrically, the follicular diameter was both a convenient and appropriate measurement for describing the cyst status; there were no differences in the thickness of each follicular layer. In conclusion, differences in the components of ECM were observed in the follicular wall of ovarian cysts compared with normal preovulatory follicles. However, since these changes did not occur uniformly in all layers of the follicular wall, their role in cyst development remains to be established.

Key words: Extracellular matrix, histochemistry, ovary, polycystic ovarian syndrome, rat

INTRODUCTION

Animal model suitable for investigating the causes and pathophysiology of the polycystic ovarian syndrome (POS) should have a clearly defined reproductive cycle. The ideal POS model should also have well-known anatomical, biological and biochemical features [22]. The laboratory rat (*Rattus norvegicus*) has been used in numerous studies, and is the species in which histological and physiological aspects of reproduction have been characterized best.

A relatively simple method for inducing POS consists of exposing mature rats to constant light [9]. Such exposure induces cysts gradually in a manner similar to that seen with spontaneous POS [36]. This model is the least invasive of the models developed so far [40,41], and can be used to investigate the etiology, pathogenesis and therapy of POS. Numerous endocrinological and biochemical studies have used polycystic ovaries induced by constant light [5,20,43]. The constant light POS model could provide useful tool for testing therapeutic and preventive measures for serious reproductive diseases in many animal species, including livestock [45].

The growth of the ovarian follicle requires extensive cellular proliferation and remodeling of the extracellular matrix (ECM) since the follicle differentiates from a small, primordial follicle with a single layer of granulosa cells to a large, preovulatory Graafian follicle [13,24]. For many years, the ovarian ECM was thought to function merely as a framework that provided the architectural support for specific ovarian structures. However, the ECM actually provides a specialized microenvironment for specific ovarian cells. The ECM composition of the preovulatory follicle wall has been described [21].

The ECM consists of fibrous and amorphous components, with the fibers forming the collagen system and the elastic system. The amorphous substance consists principally of proteoglycans (PG) which are complex macromolecules that contain a protein core with one or more covalently bound glycosaminoglycan (GAG) chains.

In this study we examined the histological structure and distribution of the ECM fibers as well as the GAG content and composition of the follicular wall of ovaries from rats with induced POS.

This paper is dedicated to the memory of our colleague Prof. Gregorio Santiago Montes.

Correspondence to: Dr. Hugo H. Ortega
Departamento de Histologia y Embriologia, Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (UNL), R. P. Kreder 2805, (3080) Esperanza, Santa Fe, Argentina. Tel: (54) (3496) 420639, Fax: (54) (3496) 426304, E-mail: hhortega@fcv.unl.edu.ar

MATERIAL AND METHODS

Animals and treatment

All the procedures were done according to the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996). Wistar rats were provided by the Center for Experimental Biology and Laboratory Animal Sciences, Faculty of Veterinary Sciences, UNL. The animals were housed on a controlled light-dark cycle (lights on from the 6:00 a.m. to 8:00 p.m.), at 20-24º C with free access to water and commercial rodent chow until used.

Twenty female virgin rats (8 weeks old 160 $\pm$ 20 g) were used. Ten rats were housed as described above except that the photoperiod was extended to 24 h (continuous light), for 15 weeks. The remaining ten rats (controls) were housed under normal conditions and were sacrificed in proestrus to obtain preovulatory tertiary follicles.

Because vaginal changes reflect the presence of POS, the rats exposed to continuous light were examined daily throughout the experiment by using vaginal smear, as described by Montes and Luque [28]. Smears obtained by vaginal washing were examined under a microscope for the relative abundance of nucleated epithelial cells, cornified cells and leucocytes. Cycles with a duration of 4 to 5 days were considered regular. The presence of cornified cells in the smears for at least 10 consecutive days was defined as persistent vaginal cornification (PVC) and was considered as confirmation of follicular cystic development [36].

Tissue sampling

The ovaries were dissected and fixed in 10% buffered formalin for 12 h, washed in phosphate buffer saline (PBS) and processed for paraffin embedding [44]. Five micrometer thick sections were mounted on glass slides pretreated with Vectabond (Vector Labs, Burlingame, USA) and stained with hematoxylin–eosin, picrosirius, Weigert's resorcin-fuchsin, PAS or alcian blue. Counter staining for nuclei was omitted.

Histochemistry

Picrosirius: Sections were deparaffinized, hydrated, and stained for 60 min in 0.2% sirius red (Direct Red 80, Aldrich, Milwaukee, WI, 53233) in saturated picric acid, followed by rapid rinsing in tap water, dehydration and mounting in natural resin [26,32].

Collagen is rich in basic aminoacids and reacts strongly with acidic dyes. Sirius red is an elongated molecule that reacts with collagen and enhances the normal birefringence of the latter by binding in parallel with the long axis of each collagen molecule. The enhanced birefringence seen with the picrosirius-polarisation method is therefore specific for collagenous structures composed of aggregates of orientated molecules [26].

Since the most probable substances that interact with the basic groups of collagen molecules *in vivo* are proteoglycans [23,33], the picrosirius method may be used in association with papain digestion, which hydrolyzes the proteic core of proteoglycans, to extracting these compounds from tissues without affecting the collagen content [38].

To study collagen organization, polarization was achieved with two Polaroid filters - one located below the condenser and the other above the objective lens of a conventional light microscope. The collagen content was quantified by observing the slides under conventional light and digitalizing the image.

Weigert's resorcin-fuchsin method: Two consecutive sections were stained with Weigert's resorcin-fuchsin, without any prior treatment or after oxidation with oxone [25,32]. This method demonstrates fully mature elastic fibers (the'*"elastic fiber"* proper) and also stains elaunin fibers (which contain less elastin). Oxytalan fibers remain unstained unless oxidized prior to staining. Oxidation was done using 10% aqueous oxone (monopersulphate compound, Du Pont, Wilmington, DE, USA).

Alcian blue: Sections were dewaxed and incubated for 18 h at room temperature in 0.05% alcian blue dissolved in 0.02 M acetate buffer, pH 5.8, containing increasing molarities (0.3, 0.65, 0.9 or 1 M) of magnesium chloride, followed by rinsing in tap water, dehydration and mounting [27,32].

The alcian blue technique, which involves the critical electrolyte concentration, is a useful procedure for identifying acidic GAGs since these polyanions have different critical electrolyte concentration points [8]. The critical electrolyte concentration is the point at which the amount of an electrolyte, such as magnesium chloride, in alcian blue solutions is sufficient to prevent staining (due to successful competition of electrolyte cations with the dye cations for binding sites in tissue polyanions). When sections are stained to equilibrium in ascending molarities of magnesium chloride in buffered solution there is a decrease in the cut off for alcian blue staining, depending on the substance present (Table 1).

Table 1. Staining reactions of glycosaminoglycans in Alcian Blue involving critical electrolyte concentration.

Glycosaminoglycans (GAGs)	$MgCl_2$ (M)			
	0.3	0.65	0.9	1.0
Non-sulphated (hyaluronic acid)	+	-	-	-
Sulphated [a]				
Chondroitin 4- and 6-sulphates	+	+	-	-
Dermatan sulphate	+	+	-	-
Heparan sulphate	+	+	+	-
Keratan sulphate	+	+	+	+

Montes and Junqueira, [27][a] Listed according to the increasing degree of sulfation.Scoring: (+) positive reaction, (-) negative reaction.

PAS (periodic acid - Schiff): Sections were deparaffinized, hydrated and immersed in 1 % periodic acid for 20 min before being treated for 20 min with Schiff reagent (Biopur®, Rosario, Argentina). The periodic acid-Schiff reaction is a widely used technique in carbohydrate histochemistry. The standard PAS method demonstrates carbohydrate components, which contain vicinal glycol groups and have a neutral electrostatic charge [18].

Image analysis

Image analysis was done using Image Pro-Plus 3.0.1® system (Media Cybernetics, Silver Spring, MA, USA). The images were

digitized by a CCD color video camera (Sony, Montvale, NJ, USA) mounted on top of a conventional light microscope (Olympus BH-2) fitted with x4, x10 and x40 objective lenses. 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 at 640 x 480 pixels. At the magnifications used, each pixel of the image corresponded to 2.68, 1.04 and 0.26 μm for x4, x10, and x40, respectively, and each field in the monitor represented a tissue area of 2.2, 0.33 and 0.02 mm^2, respectively.

Images of slides stained with alcian blue, picrosirius and PAS, were converted to an 8 bit gray scale. The intensity calibration with a gray scale between 0 (background) and 100 (black) was selected to define the area in the image that had specific staining. The optical density (OD) was measured as the mean gray score, and was equal to the sum of the OD divided by the number of pixels measured [32].

All data were expressed as the mean ± standard deviation. Statistical comparison between the controls and cystic ovaries were done using the Mann-Whitney U test. A value of $p < 0.05$ indicated significance.

RESULTS

Histology

The germinal epithelium was composed of low cuboidal cells, and an eosinophilic area between the surface epithelium and the ovarian follicles (tunica albuginea) was noticed in most of the ovaries. A striking and consistent feature was the absence of *corpora lutea* or *corpora albicans* in all of the ovaries. Numerous tertiary (Graafian) follicles and only occasional primordial or secondary follicles were present in these ovaries. The tertiary follicles were of various sizes and shapes and of irregular distribution, and were cortical as well as subcortical. Most of these follicles were markedly distended and cystic compared with the controls (Fig. 1A,B). Within the follicle, many layers of follicular cells, which collectively formed the granulosa layer and were filled with the follicular liquor, lined the antrum. Two layers of stromal cells condensed around the granulosa membrane in the cortex to form an additional coat, the follicular theca, which could be subdivided into an inner vascular theca interna and a peripheral fibrous theca externa. The granulosa cells appeared histologically normal in most ovaries. With hematoxilyn–eosin staining, theca cells usually showed a clear or light pink cytoplasm and a nucleus with dense chromatin material. The shape of the thecal cells varied from round or fusiform to epithelioid (Fig. 1C).

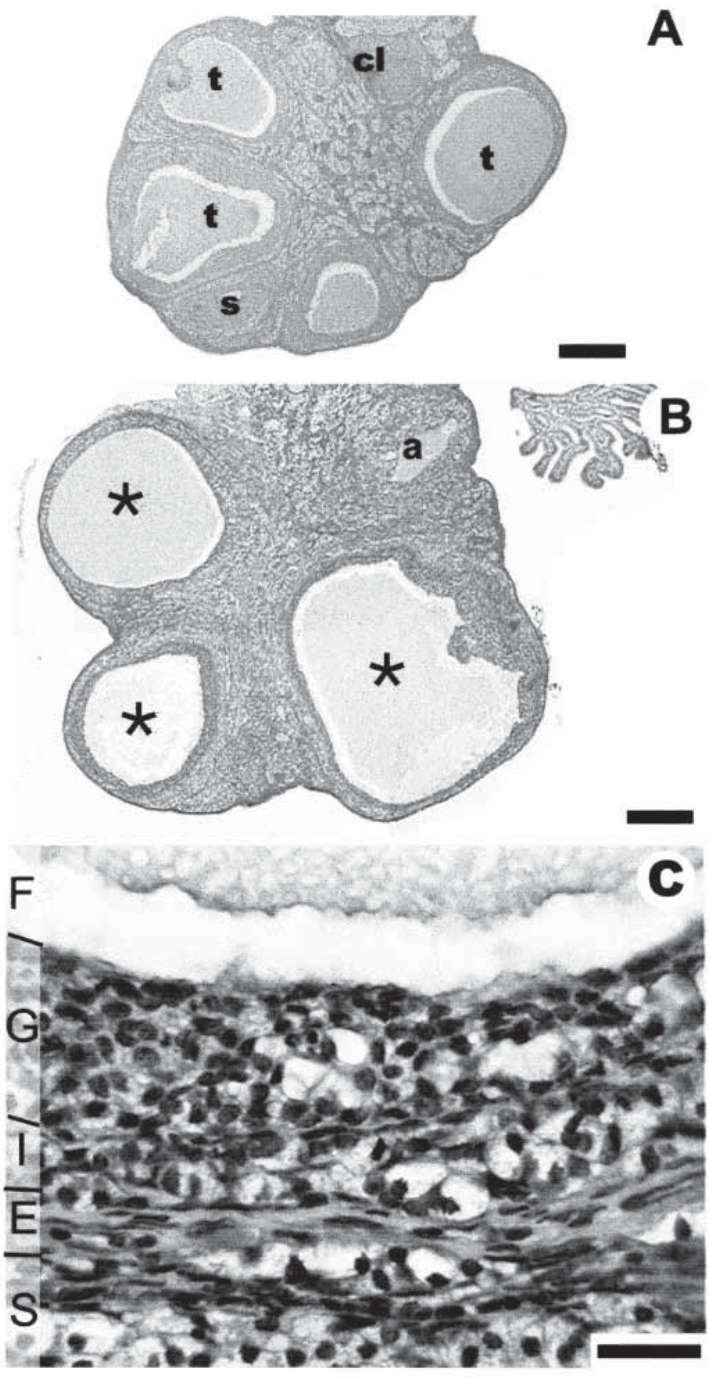

Figure 1. A) General histology of a proestrus ovary in a control rat. HE staining. Numerous tertiary (Graafian) follicles (**t**), secondary follicles (**s**) and a *corpora lutea* (**cl**) are present. Bar = 250 μm. **B)** A cystic ovary showing numerous distended (cystic) tertiary follicles (*****) and an atresic follicle (**a**). Bar = 250 μm. **C)** Detail of the follicular wall of a cystic follicle, **F** - follicular cavity, **G** - granulosa, **I** - theca interna, **E** - theca externa, **S** - stroma. Bar = 25 μm.

Collagen organization and quantification

Under polarized light, the different types of collagen that composed the follicular wall and their differential distribution were easily discernible. Type-I collagen formed thick fibers (collagen fibers), composed of closely packed thick fibrils, and consequently displayed an intense yellow to red birefringence. This fiber type was localized in the theca externa and in some areas of the theca interna around the blood vessel. Fibers formed by type-III collagen (reticulin fibers) displayed a weak, greenish birefringence and were localized in the theca externa connective tissue, in contact with the stroma or the tunica albuginea (Fig. 2A).

The quantification of the optical density in Picrosirius-stained slides, observed with conventional illumination and evaluated in the different areas of the follicular wall showed significant differences in the optical density in the theca externa of the cystic follicles (Table 2, Fig. 2B).

Distribution of the elastic system fibers

Except for the elastic tunic of the blood vessels in the theca externa (where typical, well developed, fully mature elastic fibers were seen with Weigert's resorcin-fuchsin), no elastic fibers (the "elastic fiber" proper) were detected in the follicular wall. However, both thecae were rich in elaunin and oxytalan, two types of elastic-related fibers. Elaunin fibers were localized mainly to the intercellular spaces of the theca interna and along the periphery of the bundles of collagen fibers in the theca externa (Fig. 2C). Following oxidation, Weigert's method revealed the presence of oxytalan fibers embedded in the matrix

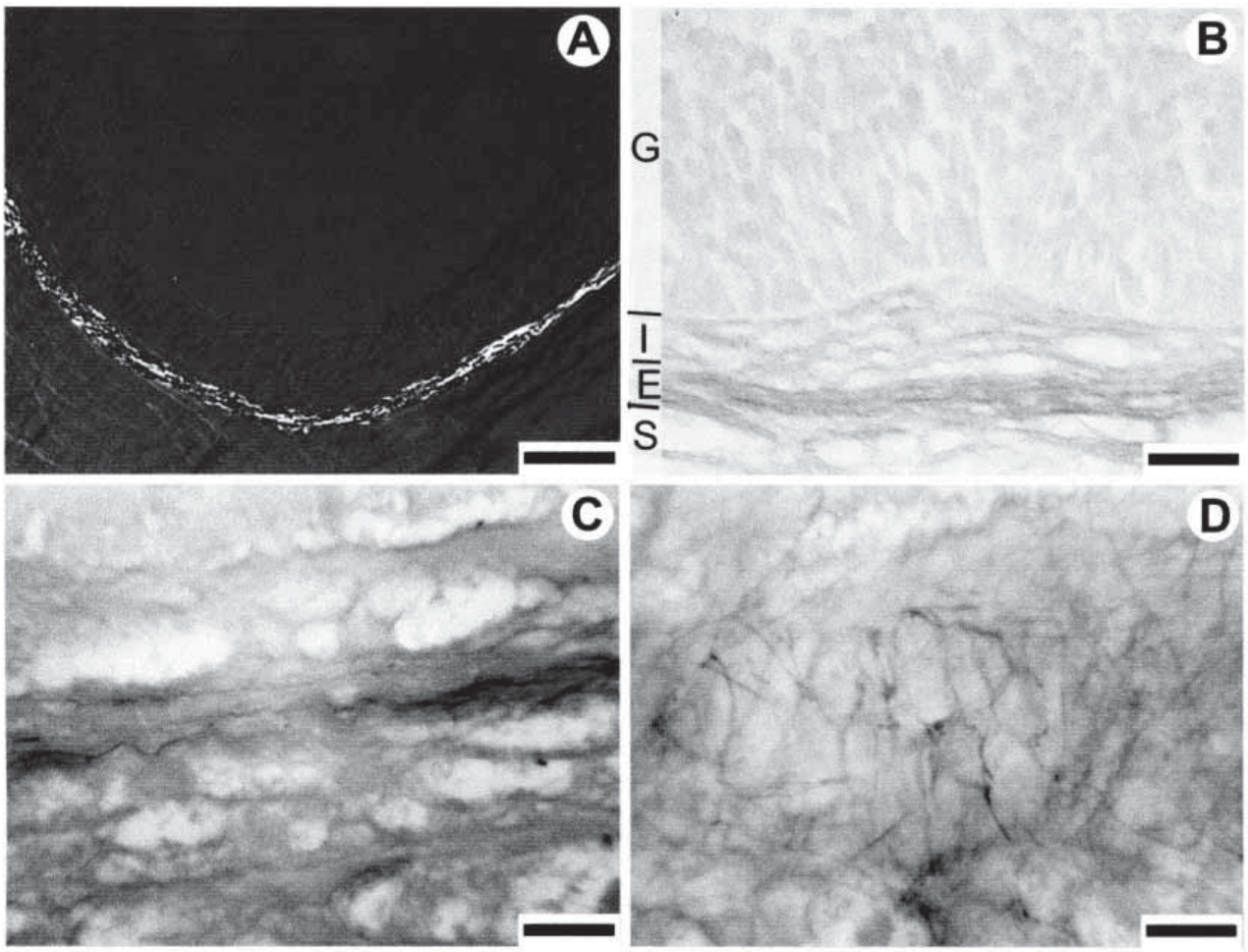

Figure 2. Extracellular matrix fibers in the wall of cystic follicles. **A)** Intense birefringence in the theca externa, corresponding mainly to type I collagen, observed with the Picrosirius-polarization method. Bar = 100μm. **B)** Detail of follicular wall stained with Picrosirius and observed with conventional light, **G** - granulosa, **I** - theca interna, **E** - theca externa, **S** - stroma. Bar = 25 μm. **C)** Elaunin fibers in the theca externa of sections stained by Weigert's method. Bar = 10 μm. **D)** Following oxidation, Weigert's method revealed oxytalan fibers embedded in the matrix of the theca externa. Bar = 10 μm.

Table 2. Quantification of the ECM components in different regions of the follicular wall using various histochemical stains.

	Picrosirius	PAS	Alcian Blue + 0.3 M $MgCl_2$
Control ovaries			
Granulosa	3.11 ± 1.35	11.82 ± 1.92 [b]	19.98 ± 3.40 [d]
Theca interna	16.93 ± 7.01	12.70 ± 5.26	3.12 ± 1.77
Theca externa	35.09 ± 7.67 [a]	24.48 ± 4.07 [c]	6.66 ± 1.49 [e]
Cystic ovaries			
Granulosa	3.78 ± 2.69	10.76 ± 1.54 [b]	10.73 ± 2.64 [d]
Theca interna	19.65 ± 7.10	14.74 ± 5.32	4.04 ± 1.83
Theca externa	50.55 ± 7.45 [a]	32.39 ± 4.21 [c]	8.96 ± 1.58 [e]

The values are the mean ± S.D. of the optical densities.Differences between control and cystic ovaries: [a, b, d, e] $p<0.01$; [c] $p<0.05$.

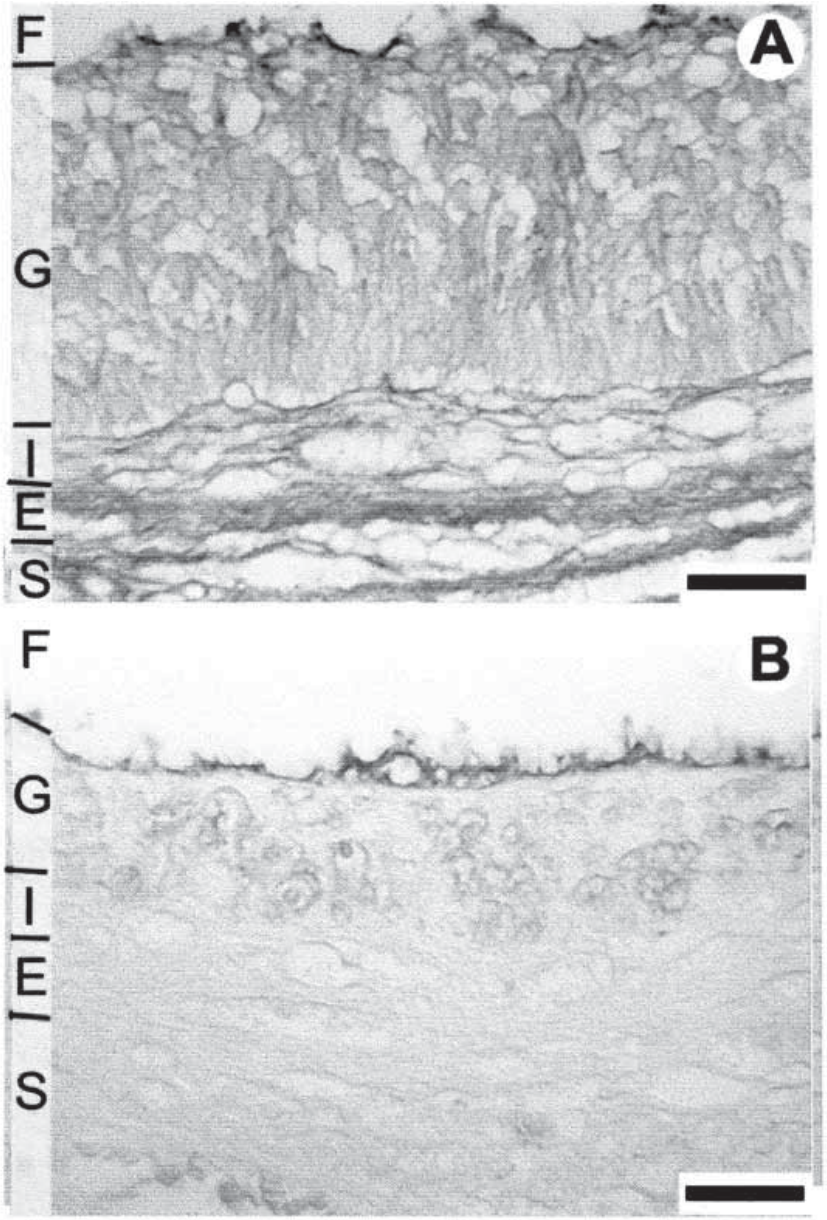

Figure 3. Amorphous substance in the ECM of the wall of cystic follicles. **A)** Localization of neutral carbohydrates with the PAS method. Bar = 25 μm. **B)** Localization of acidic GAGs by alcian blue 0.3 M $MgCl_2$ staining. Bar = 25 μm. **F** - follicular cavity, **G** - granulosa, **I** - theca interna, **E** - theca externa, **S** - stroma.

surrounding the collagen fibers in the theca externa (Fig. 2D). This distribution was similar in both, normal and cystic follicles.

PAS quantification

Neutral carbohydrates were detected in the intracellular, intercellular and interfibrous spaces stained with PAS method (Fig. 3A). Within the follicular wall, the theca externa was the most intensely stained layer, with significantly greater staining in cystic ovaries. In the granulosa, the intensity of staining was significantly lower in cystic ovaries (Table 2).

Alcian blue quantification

Acidic GAGs were detected in the intracellular, intrafibrous and interfibrous spaces stained with alcian blue (Fig. 3B). Within the follicular wall, the granulosa was the more intensely stained layer, with a significant reduction in cystic ovaries. In the theca externa the intensity of staining was significantly higher in cystic ovaries (Table 2).

Because higher optical densities were obtained at 0.3 M magnesium chloride suggested that most of the GAGs present in this tissue corresponded to hyaluronic acid. Indeed, at high molar concentrations of magnesium chloride, the optical density of the corresponding alcianophilia was hardly discernible from the background, which suggested that the more highly sulphated GAGs (chondroitin sulphate, heparan sulphate and keratan sulphate) were present at very low concentrations (if at all) in this tissue.

Histomorphometry

The histomorphometric results are presented in Table 3. The average diameter of the tertiary (or cystic) follicles was significantly greater in the cystic ovaries. The thickness of the granulosa, theca interna and theca externa did not differ significantly among the controls and cystic ovaries.

Table 3. Histomorphometric data for control and cystic ovaries (all measurements in μm).

Parameter	Control Ovaries	Cystic Ovaries
Follicle diameter	530.9 ± 99.7 [a]	736.3 ± 99.3 [a]
Follicular wall thickness	82.7 ± 28.3	84.6 ± 31.8
Granulosa thickness	54.5 ± 25.4	53.2 ± 29.8
Theca interna thickness	15.4 ± 3.3	15.6 ± 7.5
Theca externa thickness	11.8 ± 3.6[a]	11.0 ± 4.0

The values are the mean ± S.D. of parameters measured. Differences between control and cystic ovaries: [a] $p<0.01$.

DISCUSSION

The etiology and pathogenesis of the follicular cysts have been the subject of numerous hypotheses since the middle of the XIX century. However, they are not still completely clarified at the present time, neither in the bovine nor in other animal species [3,14,29].

Follicular cysts have been widely studied, particularly their diagnosis and treatment [1,6,7, 10,30,31,37]. Nevertheless, relatively little is known about the mechanisms involved in their development and their structure [15,17,34,35,45].

Folliculogenesis is characterized by proliferation of the granulosa cells, differentiation of the thecal cells from the ovarian stroma, and deposition of a basement membrane that separates the theca from avascular granulosa cells. Unlike the granulosa cells, the thecal layer is well vascularized and contains circumferential collagen bundles. The granulosa cells secrete a mucopolysaccharide-rich fluid that coalesces to form the antral cavity in the secondary follicle. The resulting mature follicle is approximately 400-fold larger than the initial primordial follicle and is surrounded by collagen and GAGs [13,24].

Changes in the ECM can influence gene expression as well as cell migration, proliferation, differentiation and apoptosis [42]. These actions of the ECM in regulating cell function are mediated by integrins, which are cell surface receptors specific for ECM components [2].

As shown here, there were histochemical differences in the granulosa and theca externa of follicular cysts of POS rats compared with normal preovulatory follicles. These histochemical changes apparently occurred in response to alterations in the regulation of follicular growth.

The theca externa is rich in type I and III collagens, which provide the follicular wall with resistance to tensile strength [39]. As shown here, the collagen content of the theca externa of follicular cysts was higher than in the controls. This finding differs from that described in other POS models [19], in which hormonal therapies can deplete ascorbic acid levels and lead to subnormal collagen synthesis [4,12].

Staining with PAS and alcian blue revealed a significantly smaller quantity of neutral carbohydrates and acid GAGs in the granulosa of cyst follicles, possibly associated with degenerative changes, as it has been described elsewhere [16]. Based on a study of the cellular regulation of FSH and the ability to

synthesize progesterone *in vitro* Erickson *et al*. [11] suggested that the granulosa cells in follicular cysts of POS in women, may be abnormal. In contrast, the strong staining seen with these methods in the theca externa, could be explained by the carbohydrates and GAGs associated with the increased amount of collagen present [18].

Histomorphometrically, the follicular diameter was a convenient and appropriate measurement for describing the cyst status, since there were no differences in the thickness of each follicular layer.

In conclusion, differences in the components of the ECM were identified and localized in the follicular wall of ovarian cysts compared with normal pre-ovulatory follicles. However, since these changes did not occur uniformly in all layers of the follicular wall, their role in cyst development remains to be established. The differential expression patterns of some of these suggest that they have key roles in follicular development.

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