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Cytoskeletal proteins in the follicular wall of normal and cystic ovaries of sows[1]

Fabiano J.F. de Sant'Ana[2]*, Janildo L. Reis Junior[2], Rosélia L.S. Araújo[2], Eduardo J.Gimeno[3] and Hugo H. Ortega[4]

ABSTRACT.- Sant'Ana F.J.F., Reis Jr J.L., Araújo R.L.S., Gimeno E.J. & Ortega H.H. 2015. **Cytoskeletal proteins in the follicular wall of normal and cystic ovaries of sows.** *Pesquisa Veterinária Brasileira 35(2):119-124.* Laboratório de Patologia Veterinária, Universidade de Brasília, Campus Universitário Darcy Ribeiro, Brasília, DF 70910-900, Brazil. E-mail: santanafjf@yahoo.com

The expression of cytoskeletal proteins was evaluated immunohistochemically in 36 normal ovaries sampled from 18 sows and 44 cystic ovaries sampled from of 22 sows, was evaluated. All sows had history of reproductive problems, such as infertility or subfertility. The immunohistochemically stained area (IHCSA) was quantified through image analysis to evaluate the expression of these proteins in the follicular wall of secondary, tertiary, and cystic follicles. Cytokeratins (CK) immunoreactivity was strong in the granulosa cell layer (GC) and mild in the theca interna (TI) and externa (TE) of the normal follicles. There was severe reduction of the reaction to CK in the GC in the cystic follicles, mainly in the luteinized cysts. The immunoreactivity for vimentin was higher in the GC from normal and cystic follicles in contrast with the other follicular structures. In the luteinized cysts, the IHCSA for vimentin was significantly higher in TI and in both observed cysts, the labeling was more accentuated in TE. Immunohistochemical detection of desmin and α-SMA was restricted to the TE, without differences between the normal and cystic follicles. The results of the current study show that the development of ovarian cysts in sows is associated to changes in the expression of the cytoskeletal proteins CK and vimentin.

INDEX TERMS: Ovarian cysts, ovary, cytoskeletal proteins, swine.

RESUMO.- [**Proteínas do citoesqueleto na parede folicular de ovários normais e císticos de porcas matrizes.**] A expressão de proteínas do citoesqueleto foi avaliada por imuno-histoquímica em ovários normais e císticos de porcas matrizes. Amostras de 36 ovários normais (18 porcas) e de 44 císticos (22 porcas) foram avaliadas. Todas as matrizes apresentaram histórico de problemas reprodutivos, como infertilidade ou subfertilidade. As áreas coradas por imuno-histoquímica (IHCSA) foram quantificadas por avaliação de imagens avaliando a expressão dessas proteínas na parede folicular de folículos secundários, terciários e císticos. A imuno-reatividade para citoqueratina (CK) foi forte na camada de células da granulosa (GC) e discreta nas tecas interna (TI) e externa (TE) dos folículos normais. Houve redução acentuada da reação de CK na CG dos folículos císticos, principalmente nos cistos luteinizados. A reação para vimentina foi mais intensa na CG dos folículos normais e císticos em comparação com outras estruturas foliculares. Nos cistos luteinizados, a IHCSA para vimentina foi significativamente maior na TI e, em ambos os cistos observados, a marcação foi mais acentuada na TE. A marcação de desmina e actina alfa de músculo liso foi restrita a TE, sem diferenças entre os folículos normais e císticos. Os resultados do presente estudo mostram que o desenvolvimento de cistos ovarianos em porcas matrizes está associado a alterações na expressão das proteínas do citoesqueleto CK e vimentina.

TERMOS DE INDEXAÇÃO: Cistos ovarianos, ovário, proteínas do citoesqueleto, porca matriz.

INTRODUCTION

Reproductive failure in sows is a common and important problem in pig herds around the world. Anestrus, irregu-

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[2] Laboratório de Patologia Veterinária (LPV), Universidade de Brasília (UnB), Brasília, DF 70910-900, Brazil. *Corresponding author: santanafjf@yahoo.com

[3] Faculdad de Ciencias Veterinárias, National University of La Plata, Calle 60 y 118, 1900 La Plata, Argentina.

[4] Faculdad de Ciencias Veterinarias, Universidad Nacional del Litoral, Calle Kreder 2805, S3080HOF, Esperanza, Argentina.

lar or prolonged estrus cycle, failure to return to heat, repeat breeding, and small litter size are some clinical signs associated to lesions in the reproductive system of sows. Ovarian cyst or cystic follicular disease (CFD) is the main reproductive disorder of sows and causes infertility and significant economic losses (Einarsson & Gustafsson 1970, Dalin et al. 1997, Heinonen et al. 1998, Schlafer & Miller 2007). In a study conducted in Finland, approximately 50% of 1708 reproductive organs of sows and gilts presented ovarian alterations and 6.2% of these lesions consisted of variable degrees of CFD (Heinonen et al. 1998).

Morphologically the ovarian cysts in sows can be small (1-2cm in diameter) and multiple. Usually, the small cysts do not present luteinization in their walls and are named cysts of the estrogenic type. The cysts can be also multiple and large. Generally, these late cysts have luteinization in their walls and are called cysts of the progesteronic type (Nascimento & Santos 2011). Multiple luteinized cysts, some with 5cm in diameter, characterize the CFD in sows (Schlafer & Miller 2007).

The pathogenesis of the cystic ovarian disease (COD) in cows has been objective of studies and researches for many years, but is still poorly understood. Some authors believe that the etiology is multifactorial (Peter 2004). In cows, the development of the cyst has been associated to many ambient, clinic, and hereditary factors (Garverick 1997, Monniaux et al. 2008). Possibly, the stress contributes with the formation of the CFD in sows (Scholten & Liptrap 1978). Some researchers cite endocrine disorders affecting the hypothalamo-hypophyseal axis related to development of the ovarian cysts in sows (Scholten & Liptrap 1978) and cows (Garverick 1997, Silvia et al. 2002, Vanholder et al. 2006). Recent investigation revealed that apoptosis and cell proliferation were altered in swine cystic follicles (Sun et al. 2012). Previous studies have demonstrated that changes and destabilization of the cytoskeletal proteins (microtubules, microfilaments and intermediate filaments) of the ovary decrease hardly the production of steroid hormones and can contribute with the cystogenesis (Chen et al. 1994). Although the expression of cytoskeletal proteins has been studied in normal ovarian structures of many species including the sheep (Marettova & Maretta 2002), cow (Van der Huck et al. 1995) and rat (Selstam et al. 1993), there are no investigations about the expression of these proteins in normal and affected swine ovary. Recently, it was suggested that cytoskeletal proteins conducting the functional and structural changes during the cystogenesis participated in the pathogenesis of the COD in rats (Salvetti et al. 2004) and in cows (Ortega et al. 2007b). These proteins contribute to the structural integrity of cells and participate in cell-to-cell binding, differentiation events, and proliferation (Luna & Hitt 1992). The cytoskeleton is composed by three distinct polymers known as microfilaments, microtubules, and intermediate filaments. Microfilaments and microtubules are homogeneous structures, whereas intermediate filaments (IF) include heterogeneous fibers, such as cytokeratins, vimentin and desmin. These IFs are found in epithelial, mesenchymal and muscle cells (skeletal, cardiac, and smooth), respectively. In addition, it is thought that, in some cases, vimentin behaves like a maturation marker and occur in cultured cells of myogenic and neurogenic origin (Salvetti et al. 2004). Smooth muscle actin (α-SMA) is a cytoskeleton protein classified into microfilaments group that is present in smooth muscle cells (Alberts et al. 1994).

This work aims to characterize by immunohistochemistry analysis the expression of cytoskeletal proteins in the follicular wall of cystic and normal ovary of sows.

MATERIALS AND METHODS

Animals and post-mortem examination. The ovaries of 40 sexually mature sows, with (n=22) and without (n=18) cysts, were used in this study. The animals were obtained from a slaughterhouse of Goiás, Brazil. Sows were submitted to the slaughterhouse due to reproductive problems, such as infertility or subfertility.

After evisceration, sections of each ovary were collected for histological and immunohistochemical analysis. The samples were fixed in 10% neutral buffered formalin for 24 h, embedded in paraffin and cut into 3-4 μm thick sections. The sections were stained with hematoxylin-eosin (HE).

Classification of follicles. Follicles were classified microscopically in primordial, primary, secondary and tertiary according to the criteria listed in Nomina Histologica (1994). Grossly, the cysts were classified in single and multiple, and histologically, in follicular and luteinized (Schlafer & Miller 2007). Eighteen follicles of the categories normal (secondary and tertiary) and affected (luteinized), and 12 follicular cysts were analyzed.

Immunohistochemistry. Details and concentration of antibodies used are summarized in Table 1. A streptavidin-biotin method was used as previously described (Salvetti et al. 2004). In brief, sections were deparaffinized, hydrated, and microwave pre-treatment (antigen retrieval) was performed by incubating the sections in 0.01M citrate buffer (pH 6). 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 18h at 4°C. The fragments were washed and then incubated for 30 min at room temperature with pre-absorbed biotinylated secondary antibodies, selected for each of the types of primary antibody used (monoclonal or polyclonal). Streptavidin-peroxidase method (BioGenex, San Ramon, CA, USA) and 3,3'-diaminobenzidine (Liquid DAB-Plus Substrate Kit; Zymed, San Francisco, CA, USA) were used as the detection system. Later, the slides were washed in distilled water and counterstained with Mayer's hematoxylin, dehydrated and mounted. Sections of skin and heart were used as positive controls. For negative controls, instead of the primary antibodies, a phosphate-buffered saline (PBS) solution was used.

Image analysis. Image analysis of the immunoreaction in tissue sections was performed by color segmentation analysis with the Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver Spring, MA, USA) (Salvetti et al. 2004, Ortega et al. 2007b). Briefly, images were digitized with a CCD-colour video camera (Leica ICC50 HD, Heerbrugg, Switzerland) mounted on a conventional light microscope (Leica DM500, Heerbrugg, Switzerland), with an

Table 1. Panel of antibodies used in the current study

Antibody specificity	Source	Clone	Dilution
Alpha smooth muscle actin (α-SMA)	Novocastra	αsm-1	1:50
Desmin	Zymed	H2	Prediluted
Cytokeratin (Pan AE1/AE3)	Dako	H94	1:500
Vimentin	Zymed	H9	1:100

objective magnification of X40. The details of image analysis as a valid method for quantifying expression, and the methodological details, were described previously (Ortega et al. 2004). 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 color tagged image file format (TIFF).

To obtain quantitative data regarding immunohistochemical reactivity in the follicular wall, at least three sections for each specimen and antibody were evaluated and 45 representative fields were analyzed. By means of AutoPro macro language, an automated sequence operation was created to measure the immunohistochemically stained (labelled) area (IHCSA). IHCSA was calculated as a percentage of total area evaluated by color segmentation analysis, which extracts objects by locating all objects of a specific color (brown). The brown label was selected and a mask was then applied to make the separation of colors 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, Ortega et al. 2007a). The major strength of the well-validated imaging approach used in this study is the visualization of in situ localization of proteins within cells of interest. Quantification of biological markers using this approach has been successfully applied to quantify immunoreactivity in different tissues (Lejeune et al.2008, Ortega et al. 2009a). This type of densitometrical methodology has been previously validated by biochemical methods of protein induction and quantification (Peretti-Renucci et al. 1991).

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 analyzed by means of the ANOVA test and the Duncan post-test.

RESULTS

Morphology

Thirty-six ovaries (18 animals) do not showed morphologic alterations, exhibiting follicles in different stages, including primordial, primary, secondary and tertiary follicles, corpus luteum and corpora albicans. Macroscopic and microscopic examination showed that 44 ovaries (22 sows) presented cysts (2.2-4.0cm in diameter). In the majority of these cases (42/44), the cysts were multiple, bilateral and occupied almost the entire ovarian parenchyma. The multiple follicular cysts were characterized for a large antral cavity and atrophy and disorganization of the granulosa cell (GC) layers and of theca interna (TI) and externa (TE). The multiple luteinized cysts were similar to follicular cysts, but there was no GC or this layer was severely atrophied and hyalinized. Furthermore, there was moderate to severe luteinization of the TI and TE. Only two ovaries presented single luteinized cyst and these cases was not considered for evaluation.

Immunohistochemistry

The immunohistochemical expressions of the used antibodies are shown in Table 2. The immunoreactions were negative in all negative controls and positive in the positive controls.

In the follicular wall, the labeling to α-SMA was restricted to TE and negative in the TI and CG (Fig.1). There was no significant differences among the IHCSAs of the normal and cystic follicular structures (P>0.05). Smooth muscle cells including those present in the cortical and medullar blood vessel walls functioned as internal control to α-SMA.

Similar to immunohistochemical reaction observed with α-SMA, the reactivity for desmin was limited to TE (Fig.2), without differences between the normal and cystic follicles (P>0.05). Some interstitial mesenchymal cells of the medullary and cortical stroma and the blood vessel wall showed moderate to strong positive reaction for desmin.

In all normal and cystic follicles, there was positive reaction to CK. CK immunoreactivity was strong in the GC and mild in the TI and TE of the secondary and tertiary follicles (Fig.3A and 3B) (P<0,05). There was severe reduction of

Table 2. Mean and standard error of the immunohistochemically stained area (IHCSA) by cytoskeletal proteins in follicular structures of normal and cystic ovaries of sows

Antibody	Secondaryfollicle	Terciaryfollicle	Follicularcyst	Luteinizedcyst
α-SMA				
GC	-	-	-	-
TI	-	-	-	-
TE	11.32+2.14a	10.85+1.89a	10.62+3.31ª	12.18+2.52a
Desmin				
GC	-	-	-	-
TI	-	-	-	-
TE	8.26+2.59a	7.11+2.1a	6.75+1.83ª	5.62+1.2a
CK				
GC	10.89+2.55aA	8.39+0.47aA	2.74+1.35bA	0.35+0.25bA
TI	1.64+0.83aB	0.78+0.39aB	1.57+1.16aA	3.38+1.82bB
TE	0.69+0.39aB	-	0.87+0.72aA	0.98+0.5aA
Vimentin				
GC	9.41+2.35aA	9.77+2.71aA	10.98+1.01aA	8.64+2.44aA
TI	3.9+1.76aB	7.88+2.05aA	4.66+1.97aB	16.86+5.01bB
TE	2.33+0.9aB	4.63+0.21bB	9.91+1.92cA	12.03+2.6cA

α-SMA = α-Smooth Muscle Actin, CK = cytokeratin, GC = granulosa layer, TI = theca interna, TE = theca externa. In horizontal rows, means followed of distinct lowercase letters differ significantly (P<0,05). In columns, means followed of distinct capital letters differ significantly (P<0,05).

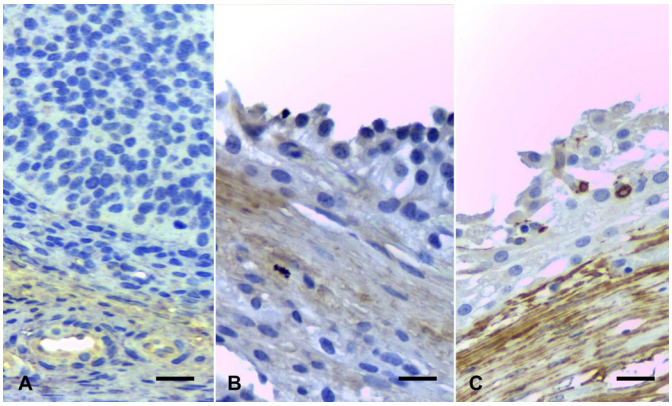

Fig.1. (**A**) Immunohistochemical localization of alpha smooth muscle actin in secondary follicles, (**B**) follicular cyst, and (**C**) luteinized cyst is restricted to the theca externa. Bars = 24μm.

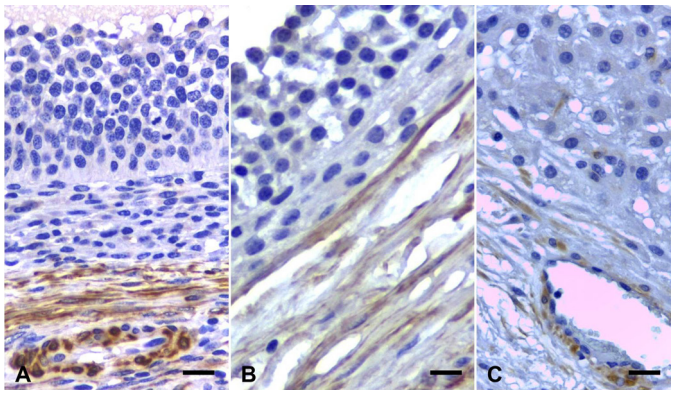

Fig.2. (**A**) Immunohistochemical reactivity to desmin in secondary follicles, (**B**) follicular cyst and (**C**) luteinized cyst is restricted to theca externa. Bars = 24μm

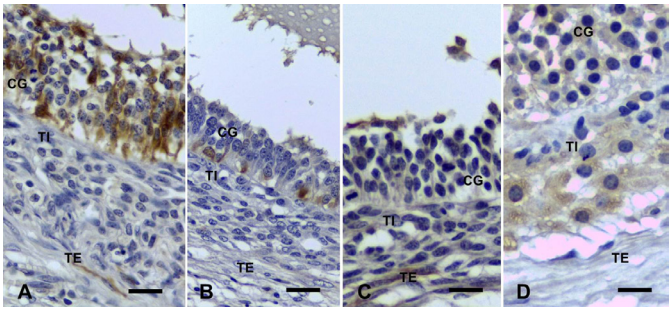

Fig.3. Immunohistochemical reaction to cytokeratin (CK) (**A**) in secondary and (**B**) tertiary follicles, and (**C**) in follicular and (**D**) luteinized cysts. The immunoreactivity is strong in the granulosa cell layer (CG) and mild in the thecasinterna (TI) and externa(TE) of the secondary and tertiary follicles. Note severe reduction of the marcation to CK in the GC of the cystic and luteinized follicles. Bars = 24μm.

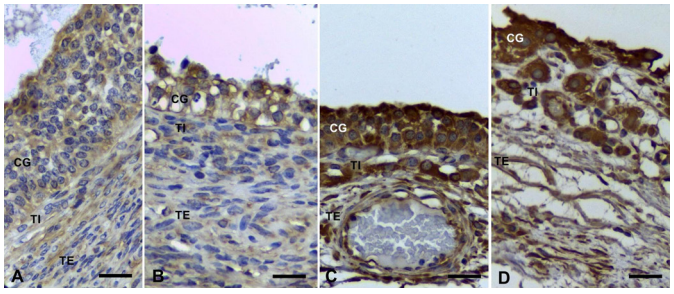

Fig.4. Immunohistochemical demonstration of vimentin (**A**) in secondary and (**B**) tertiary follicles, and (**C**) follicular and (**D**) luteinized cysts. The reaction is higher in the granulosa cell layer (GC) of normal and cystic follicles in contrast with the other follicular layers. In the luteinized cysts, the IHCSA for vimentin was significantly higher in theca interna(TI) and in both observed cysts, the labeling was more accentuated in theca externa (TE). Bars = 30μm.

the binding to CK in the GC of the cystic follicles, mainly in the luteinized cysts (Fig.3C and 3D) (P<0,05). A reaction in the TI of the luteinized cysts was significantly higher in contrast to the other follicular structures (P<0,05). In addition, strong reactivity in the cells of the GC of the primordial follicles and follicular fluid was noted. The epithelium of ovarian surface presented homogeneous and strong reaction to CK, functioning as positive internal control.

In all normal and cystic follicles, there was positive labeling to vimentin. The immunoreactivity for this antibody was higher in the GC of normal and cystic follicles in contrast with the other follicular structures (Fig.4) (P<0,05). GC of the primordial follicles and some mesenchymal cells presented moderate positive reaction. In the luteinized cysts, the IHCSA for vimentin was significantly higher in TI (P<0,05) and in both observed cysts, the labeling was more accentuated in TE (P<0,05).

DISCUSSION

Although cystic ovaries is considered a common and important economically reproductive disease of domestic animals, the mechanisms that leading to development of these cysts have been object of speculation and research for many years, but are not fully understood (Wiltbank et al. 2003). Many studies have demonstrated changes and pathogenetic mechanisms evolved in follicular cyst in cattle (Garverick 1997, Isobe & Yoshimura 2007, Ortega et al. 2007b, Monniaux et al. 2008, Ortega et al. 2008, Rey et al. 2010), rat (Anderson & Lee 1997, Ortega et al. 2007a, Salvetti et al. 2009) and women (Abbot et al. 2002, Ortega et al. 2009b), but few investigations have been conducted in the genesis of this condition in sows. The current study shows that the formation of ovarian cysts in sows is associated to alterations in the expression of some cytoskeletal proteins. These molecules act in the formation of cell contacts and in the determination of the cell morphology (Schliwa & Van Blerkom 1981, Luna & Hitt 1992). Changes in the intermediate filaments have also been observed in ovine normal ovaries, affecting the organization of the cytoskeleton of corpus luteum and atresic follicles (Marettova & Maretta 2002). In addition, studies have demonstrated that pharmacologic agents which destabilize the cytoskeletal proteins can affect considerably the production of steroid hormones (Chen et al. 1994), determining important changes in the steroidogenesis and consequently in cystogenesis (Isobe et al. 2003).

In the current investigation, no significant alterations in the areas of immunoreaction for desmin and α-SMA between the normal and cystic follicles were found, suggesting that these expressions are restrict the stable structures that either do not participate or are not changed during the cystogenesis. Furthermore, the reaction for these antibodies was limited to TE. Very similar dates were observed in previous studies conducted in cows (van der Hurk et al. 1995, Ortega et al. 2007b) and rats (Salvetti et al. 2004). Desmin and α-SMA are important antibodies used to labeling of muscle cells in general (skeletal, cardiac, and smooth) and smooth muscle cells, respectively, in normal and pathological situations. Alterations in the ovarian immunoreaction of these antibodies can indicate stromal disturbs related to formation of lesions, such as cysts.

The CK reactivity was more marked in GC and discrete in the TI and TE of the healthy follicles, as observed in normal follicles of cows (Ortega et al. 2007b). However, in the current study, there was significant reduction of the IHCSAs for GC in the two types of analyzed cysts. On the other hand,

the expression of CK in the GC was increased in rats and cows with ovarian follicular cysts (Salvetti et al. 2004, Ortega et al. 2007b). These results demonstrate the importance of the animal species in relation the changes related to reactivity of cytoskeletal proteins in ovarian structures. Furthermore, in the ovaries of the current investigation, there was atrophy of the epithelium of the GC in many cysts. This fact could contribute with the reduced expression of CK. These IF is found regularly in epithelial cells and present wide diversity of subunits (Alberts et al. 1994).

The immunoreactivity for vimentin was stronger in GC in comparison to TI and TE in the normal follicles. In experimental and natural studies of cystic ovaries performed in rats (Salvetti et al. 2004) and cows (Ortega et al. 2007b), there was similar expression for this intermediate filament in the TI and GC. The pathologic date of the present study are partially similar to noted previously in bovine ovarian cysts, that also observed higher IHCSA for vimentin in the TE of follicular cysts (Ortega et al. 2007b). In addition, the strong reaction of this protein in the TI of the luteinized cysts of sows was attributed to numerous positive luteinized cells present in this affected layer. Vimentin occur in many cells of mesodermic origin, such as fibroblasts, endothelial cells and other mesenchymal cells, but is also expressed transitory in many cells during the development (Alberts et al. 1994).

CONCLUSION

The results of the current study suggest that changes in the reactivity to vimentin and cytokeratin observed in the wall of follicular and luteinized cysts in sows, probably reflect structural and functional alterations during the cystogenesis, as noted in cows (Ortega et al. 2007b) and rats (Salvetti et al. 2004).

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