Reproduction in Domestic Animals

Reprod Dom Anim **50**, 327–332 (2015); doi: 10.1111/rda.12495
ISSN 0936–6768

Immunohistochemical Expression of Growth Factors in the Follicular Wall of Normal and Cystic Ovaries of Sows

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Contents
The expression of growth factors was evaluated immunohistochemically in normal and cystic ovaries of sows. The immunohistochemically stained area (IHCSA) was quantified by image analysis to analyse the expression of these proteins in the follicular wall of secondary, tertiary and cystic follicles. IGF-I immunoreactivity was strong in the granulosa cell layer (GC), moderate in the theca interna (TI) and mild in the theca externa (TE) of the normal follicles. There was severe reduction of the labelling to IGF-I in the GC of the follicular and luteinized cysts. In the normal follicles, the reactivity for IGF-II was very similar to pattern noted in IGF-I. There was reduction of the IHCSAs in the GC of the follicular and luteinized cysts, but the decrease was not significant. The staining of the IGF-II in the TI and TE of the cysts was increased, in comparison with normal follicles. The IHCSAs for VEGF were higher in the GC and TE of the normal follicles in contrast to TI, but this difference was noted only in the tertiary follicle. The VEGF reactivity increased in the GC of the cysts, in relation to normal follicles. The results of the current study show that the formation of ovarian cysts in sows is associated with alterations in the immunohistochemical expression of some growth factors.

Introduction

Reproductive failure in sows is a common and important problem in porcine herds around the world. Anestrus, irregular or prolonged oestrous cycle, failure to return to heat, repeat breeding and small litter size are some clinical signs associated with lesions in the reproductive system of sows. Ovarian cyst is the main reproductive disorder of sows and causes infertility and significant economic losses (Einarsson and Gustafsson 1970; Dalin et al. 1997; Heinonen et al. 1998; Schlafer and 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 were ovarian cysts in variable degrees (Heinonen et al. 1998).

The pathogenesis of the ovarian cysts in bovine has been the objective of studies and researches for many years, but is still poorly understood. Some authors believe that the aetiology is multifactorial (Peter 2004). Possibly, the stress contributes with the formation of the cysts in sows (Scholten and Liptrap 1978). In cows, the development of the cyst has been associated with many ambient, clinic and hereditary factors (Garverick 1997; Monniaux et al. 2008). Some researchers cite endocrine disorders affecting the hypothalamo-hypophyseal axis related to development of the ovarian cysts in sows (Scholten and Liptrap 1978) and cows (Garverick 1997; Silvia et al. 2002; Vanholder et al. 2006). Recent investigations revealed that apoptosis and cell proliferation (Sun et al. 2012) and the expression of cytoskeletal proteins (Sant'Ana et al. 2015) were altered in swine cystic follicles. Previous studies demonstrated that insulin-like growth factors (IGFs) are produced and act in the granulosa cells of bovine normal ovaries (Ortega et al. 2008) and that in cystic ovaries occur a decrease in the production of growth factors in cows (IGF-I and IGF-II) and rats [IGF-I and vascular endothelial growth factor (VEGF)] (Ortega et al. 2007a, 2008; Rey et al. 2010). Investigations have demonstrated that IGF-I stimulates the production of oestrogen for the granulosa cells, increase the induction of receptors to LH, improves the induction of the activity of aromatase by FSH and induce the proliferation and differentiation of granulosa cells and of the thecas (Adashi et al. 1985; Giudice 1992). IGF-II act also positively in the folliculogenesis, mainly on the thecal cells steroidogenesis, proliferation and differentiation of follicles (Spicer et al. 2004). Vascular endothelial growth factor may act in the regulation of the follicle growth and in determine which follicle becomes dominant during the follicular selection process (Danforth et al. 2003).

This work aims to characterize by immunohistochemistry the expression of growth factors in the follicular wall of cystic and normal ovary of sows.

Material 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.

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 haematoxylin–eosin (HE).

Classification of follicles

Follicles were classified microscopically in primordial, primary, secondary and tertiary according to the criteria

listed in the Nomina Histologica (1994) and Priedkalns and Leiser (2006). Follicles were considered primordial when had an oocyte surrounded by a simple squamous epithelium of follicular cells. Primary follicles were composed of an oocyte surrounded by a simple cuboidal epithelium of follicular cells. Secondary follicles had an oocyte surrounded by a stratified epithelium of polyhedral follicular cells (granulosa cells), and tertiary follicles were composed of an oocyte surrounded by a stratified epithelium of granulosa cells; these last cells were surrounded by a multilaminar layer of specialized stromal cells (theca) and a fluid-filled cavity (antrum). Grossly, the cysts were classified in single and multiple, and histologically, in follicular and luteinized (Schlafer and Miller 2007). Cysts classified as follicular had diameter higher than 1.1 cm and consisted of a large antrum and atrophy and degeneration of the granulosa cell (GC) layers and of the thecas interna (TI) and externa (TE). Luteinized cysts were similar to follicular cysts, but there was no GC or this layer was severely atrophied and hyalinized. In addition, these cysts range from 1.1 to 5 cm and have marked luteinization of the thecas.

Eighteen follicles of the categories normal (secondary and tertiary) and affected (luteinized), and 12 follicular cysts were analysed.

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 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 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 haematoxylin, dehydrated and mounted. Sections of skin and heart were used as positive controls (Ortega et al. 2008). 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 colour 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), 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 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).

Table 1. Panel of antibodies used in the current study

Antibody specificity	Source	Dilution
IGF-I	Biorbyt	1 : 100
IGF-II	Biorbyt	1 : 100
VEGF	Biogenex	1 : 30

IGF-I, insulin-like I growth factor, IGF-II, insulin-like II growth factor, VEGF, vascular endothelial growth factor.

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 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). 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; 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. 2009). 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 analysed by means of the ANOVA test and the Duncan post-test. Previously, the distribution of data was tested for normality and equal variance using the Kolmogorov–Smirnov test. Statistical significance was accepted at $p < 0.05$.

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 (2.2–4.0 cm 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 the thecas 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 ovary presented single luteinized cyst and these cases were not considered for evaluation.

Immunohistochemistry

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

IGF-I presented strong reaction in the GC, moderate in the TI and mild in the TE of the secondary and tertiary follicles ($p < 0.05$). There was considerable reduction of the labelling in the GC of the follicular and luteinized cysts ($p < 0.05$) (Fig. 1). IGF-I reactivity was reduced in the TI of the follicular cysts ($p < 0.05$).

In the normal follicles labelled with IGF-II, the reactivity was similar to pattern noted in IGF-I with severe reaction in the GC, moderate in the TI and mild in the TE ($p < 0.05$). There was reduction of the IHCSAs in the GC of the follicular and luteinized cysts, but the decrease was not expressive ($p > 0.05$). The staining of the IGF-II in the TI and TE of the cysts was increased, in comparison with normal follicles ($p < 0.05$) (Fig. 2).

The IHCSAs for VEGF were higher in the GC and TE of the normal follicles in contrast to TI, but this difference was significantly noted only in the tertiary follicle. The reactivity increased in the GC of the cysts, in relation to normal follicles ($p < 0.05$) (Fig. 3).

Discussion

Although ovarian cyst is considered a common and important economically reproductive disease of domestic animals, the mechanisms leading to the 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 alterations and pathogenetic mechanisms evolved in follicular cyst in cattle (Garverick 1997; Isobe and Yoshimura 2007; Ortega et al. 2007b, 2008; Monniaux et al. 2008; Rey et al. 2010), rat (Anderson and Lee 1997; Ortega et al. 2007a; Salvetti et al. 2009) and women (Abbott et al. 2002; Ortega et al. 2009), 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 immunohistochemical expression of some growth factors.

Numerous studies show the participation of growth factors in the physiologic and pathologic ovarian regulation (Hunter et al. 2004). For example, changes in the IGFs system of the dominant follicle act as critic determinant of the follicular dominance (Fortune et al. 2004). There are evidences that IGFs can be regulators of the follicular growth and increase the action of the gonadotropins in the ovary (Giudice 1992). In addition, VEGF acts in the regulation of the growth and in the follicular dominance (Danforth et al. 2003). However,

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

Antibody	Secondary follicle	Tertiary follicle	Follicular cyst	Luteinized cyst
IGF-I				
GC	15.1 ± 2.69aA	13.34 ± 1.89aA	4.05 ± 0.87bA	6.38 ± 2.47bA
TI	3.01 ± 1.23aB	2.91 ± 0.96aB	1.54 ± 0.13bB	6.6 ± 3.72aA
TE	0.57 ± 0.46aC	0.85 ± 0.28aC	0.48 ± 0.21aC	0.68 ± 0.29aB
IGF-II				
GC	9.89 ± 2.78aA	10.6 ± 2.45aA	7.74 ± 2.91aA	6.47 ± 2.3aA
TI	1.64 ± 0.73aB	3.38 ± 2.19aB	4.93 ± 2.58bB	8.91 ± 2.36bA
TE	0.36 ± 0.12aC	0.63 ± 0.24aC	2.59 ± 1.83bB	3.06 ± 1.79bB
VEGF				
GC	8.36 ± 2.23aA	8.15 ± 1.11aA	11.82 ± 1.77bA	12.28 ± 2.04bA
TI	4.76 ± 3.49aA	1.84 ± 0.22bB	2.89 ± 1.23aB	5.64 ± 2.81aB
TE	11.2 ± 3.2aA	6.2 ± 1.89bA	12.81 ± 2.27aA	16.04 ± 2.6aA

IGF-I, insulin-like I growth factor, IGF-II, insulin-like II growth factor, VEGF, vascular endothelial growth factor, GC, granulosa cells 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 ($p < 0.05$).

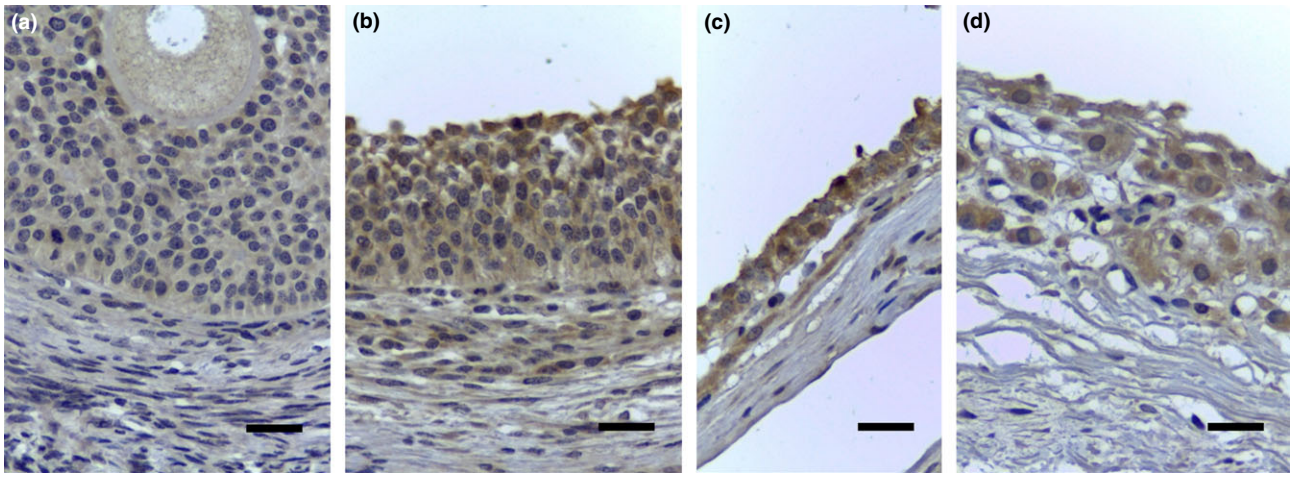

Fig. 1. Immunohistochemical reaction to IGF-I in secondary (a) and tertiary (b) follicles and follicular (c) and luteinized (d) cysts. The immunoreactivity is strong in the granulosa cell layer (GC), moderate in the theca interna (TI) and mild in the theca externa (TE). Note severe reduction of the labeling in the GC of the cysts. Bars = 24μm.

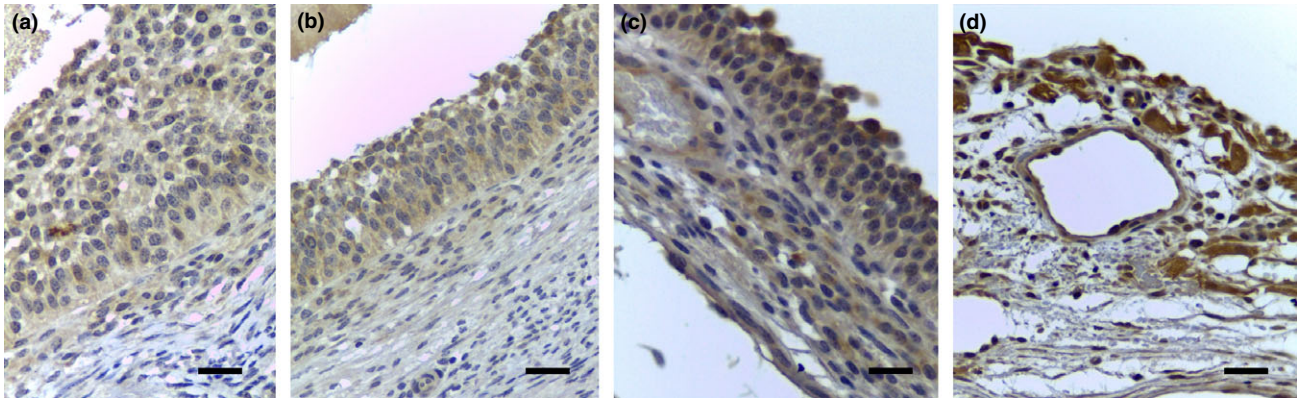

Fig. 2. Immunohistochemical reactivity to IGF-II in secondary (a) and tertiary (b) follicles and follicular (c) and luteinized (d) cysts. The reaction is strong in the granulosa cell layer (GC), moderate in the theca interna (TI) and mild in the theca externa (TE). Note mild reduction of the IHCSAs in the GC of the cysts.

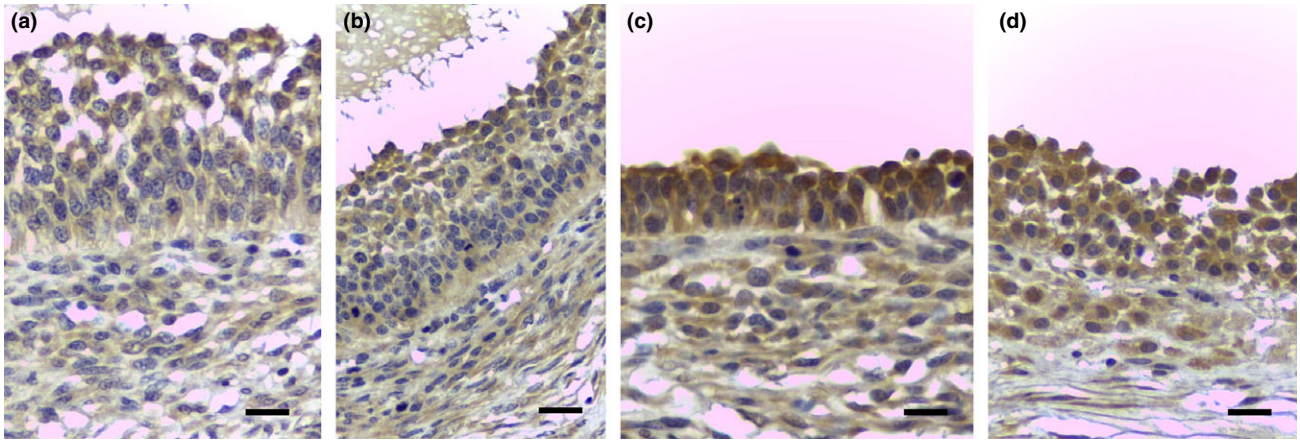

Fig. 3. Immunohistochemical demonstration of VEGF in secondary (a) and tertiary (b) follicles and follicular (c) and luteinized (d) cysts. The reactivity is higher in the GC and TE of the normal follicles, mainly in the tertiary follicle. The VEGF labeling is increased in the CG of the cysts.

limited data of the specific localization of growth factors related to ovarian changes in livestock animals have been reported (Ortega et al. 2008; Rey et al. 2010). Previous studies suggest that abnormalities in the systems of the growth factors can be involved in the pathogenesis of the cystic ovarian disease (COD) in

humans (De Leo et al. 2000; Hammadeh et al. 2003) and in animals (Dyck et al. 2001).

In the current investigation, the IGF-I and IGF-II reactivity was more marked in GC of the secondary follicles, as previously observed in rats (Ortega et al. 2007a; Rey et al. 2010) and cows (Ortega et al. 2008). There was severe reduction of the expression of IGF-I in GC in the follicular and luteinized cysts and in the TI in the follicular cysts. Similar results with intense reduction of immunomarcation in all layers of cystic follicles were noted in rats (Ortega et al. 2007a). In induced and spontaneous bovine follicular cysts, there was also reduction of expression of IGF-I in GC and TI (Ortega et al. 2008). As IGF-I intensify the physiologic induction of LH receptors (Adashi et al. 1985) and there was reduction of this protein in GC and TI in the cysts of the current study, our results suggest involvement of IGF-I in the genesis of ovarian cysts in sows, as was speculated in cows (Ortega et al. 2008). IGF-I concentrations were also reduced in the follicular fluid of bovine ovarian cysts (Ortega et al. 2008). In contrast to data obtained in the current study, the reactivity to IGF-II increased significantly in cystic follicles of cows (Rey et al. 2010). In the present investigation, IGF-II expression was increased in TI and TE of the analysed cysts, whereas immunoreactivity to this growth factor was amplified in the three layers of the follicular cysts (Rey et al. 2010). Studies have demonstrated the benefic effects of IGF-II in the steroidogenesis, proliferation and ovarian follicular differentiation (Spicer et al. 2004); however, few investigations have been performed to evaluate the participation of this protein in ovarian lesions.

The immunoreactivity for VEGF was more expressive in TE and GC in comparison with TI in the normal follicles, whereas the cysts increased the IHC-SAs in TE and GC. Similar data were observed in cystic and normal ovarian follicles of rats (Ortega et al. 2007a). Expression pattern to VEGF in the follicular structures of normal human ovaries was comparable to data obtained in the current study (Gordon et al. 1996).

The results of the current study suggest that changes in the expression of growth factors deregulate the follicular dynamic and can be involved in the pathogenesis of ovarian cysts in sows.

Acknowledgements

The authors would like to thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil (Proc. 471907/2011-1) for their financial support of this project and the Frigorífico BRF S.A. (Rio Verde, Goiás, Brazil) for provide the ovary samples used in the study. E.J.G. and H.H.O. are Research Career Members of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

FJF Sant'Ana, GR Blume and JL Reis Junior designed the study, analysed the data and drafted the manuscript. EJ Gimeno, F Rey and HH Ortega analysed the data and revised the manuscript.

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Submitted: 10 Oct 2014; Accepted: 13 Jan 2015

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