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SPONTANEOUSLY ARISING DISEASE

Role of Components of the Insulin-like Growth Factor System in the Early Stages of Ovarian Follicular Persistence in Cattle

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Summary

Cystic ovarian disease (COD) is one of the main causes of infertility in dairy cattle. It has been postulated that the insulin-like growth factor (IGF) system may contribute to follicular persistence and development of COD. The initiation of the IGF response is a result of interactions between IGF-binding proteins (IGFBPs) and IGFBP proteases, mainly pregnancy-associated plasma protein A (PAPP-A). IGFBPs bind IGFs with high affinity and consequently regulate their access to IGF receptors (IGFRs). The aim of this research was to determine variations in components of the IGF system in the ovaries of cows with persistent follicles induced by long-term administration of progesterone. Proteins of the IGF system were evaluated at 0 (expected day of ovulation), 5, 10 and 15 days of follicular persistence to determine whether the changes occur early in the development of COD. The concentrations of IGF1 and IGFBP4 in follicular fluid were similar in all groups with follicular persistence and in control antral follicles. IGFR1 and IGFBP4 expression *in situ* were higher in granulose cells in persistent follicles than in control follicles. No differences were found in PAPP-A concentration within follicular fluid in persistent follicles relative to control antral follicles. These data support the hypothesis that the IGF system is altered in the initial stages of development of follicular persistence and has a determinant role in ovarian function in cattle.

Keywords: cattle; follicular persistence; insulin-like growth factor system; progesterone

Introduction

In recent years, reproductive disorders during the early postpartum period have led to great economic losses (Lucy, 2008). Cystic ovarian disease (COD) is a major disorder contributing to poor reproductive efficiency of lactating dairy cows (Kesler and Garverick, 1982). COD is characterized by the presence of large follicular structures that persist in the ovary for 10 days or more in the absence of a corpus luteum, with interruption of the normal oestrous cycle (Silvia *et al*., 2002; Thomas *et al*., 2007). The most widely accepted hypothesis postulates that COD is the result of a 'hormonal imbalance' within the hypothalamic–pituitary–gonadal axis (Vanholder *et al*., 2006); however, experimental evidence also suggests that follicular persistence may be caused by intra-ovarian components (Ortega *et al*., 2015). In this sense, different physiological alterations, such as a delay in the first ovulation and/or in the pre-ovulatory luteinizing hormone (LH) surge, with alterations in LH pulses, have been demonstrated (Opsomer *et al*., 1999; Díaz *et al*., 2015).

Although the exact mechanism by which COD occurs is not defined, the influence of various metabolic and hormonal factors is suggested. Of these factors, gonadotropins and the insulin-like growth factor (IGF) system have been proposed as key mediators

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of ovarian dysfunction and development of COD (Spicer and Chamberlain, 2000; Vanholder *et al.*, 2006; Thomas *et al.*, 2007; Ortega *et al.*, 2008; Rey *et al.*, 2010; Rodríguez *et al.*, 2011, 2013, 2015). The IGF system is an important regulator of follicular development and selection, cell differentiation, steroidogenesis and oocyte maturation (Giudice, 1992; Spicer and Echternkamp, 1995; Brogan *et al.*, 2010). This system is composed of two ligands (IGF1 and IGF2), specific receptors (IGFR1 and IGFR2), six IGF-binding proteins (IGFBP1 to 6) and IGFBP proteases (Spicer and Echternkamp, 1995; Silva *et al.*, 2009; Sanchez *et al.*, 2014). IGFs are involved in proliferation and follicular development, while IGFBPs are responsible for increasing the average life of these ligands, forming inactive complexes, as well as for transport to the site of action (Monget *et al.*, 2002; Brogan *et al.*, 2010). IGFR1 is mainly involved in the actions of IGF1 (Monget *et al.*, 2002) and IGFBP specific proteases cleave IGFBPs, releasing IGFs (Spicer, 2004; Conover, 2012; Oxvig, 2015).

The main protease detected in cattle is pregnancy-associated plasma protein A (PAPP-A) (Mazerbourg *et al.*, 2001; Spicer, 2004; Conover, 2012). PAPP-A is responsible for cleavage of IGFBP4 in ovarian follicular fluid (FF) (Conover *et al.*, 1999) and may contribute to the bioavailability of free IGF1 for follicle development to the pre-ovulatory phase (Monget *et al.*, 2003; Spicer, 2004; Aad *et al.*, 2009; Sudo *et al.*, 2007).

In previous studies, we observed modifications in the IGF system in cows with COD (Ortega *et al.*, 2008; Rey *et al.*, 2010; Rodríguez *et al.*, 2011, 2013, 2015). Therefore, the aim of the present study was to determine the concentrations of IGF1, IGFBP4 and PAPP-A in FF, and IGFBP4 and IGFR1 protein expression in bovine ovarian follicles at different stages of persistence. Since these members of the IGF system have been suggested to be the main components modified in cows with COD, we aimed to determine the critical moment of altered expression and gain insights into the role of these members of the IGF system in COD pathogenesis, by using a previously optimized model of bovine follicular persistence, proven to be useful to study the early stages of cyst formation in cattle (Díaz *et al.*, 2015, 2016).

Materials and Methods

Ethical Approval

All procedures were carried out according to the Guide for the Care and Use of Agricultural Animals in Research and Teaching (Federation of Animal Science Societies, 2010) and the protocol was approved by the Ethics and Safety Committee of the Facultad de Ciencias Veterinarias of the Universidad Nacional del Litoral, Santa Fe, Argentina, under protocol number 131/12.

Animals and Experimental Design

The study was performed using non-lactating Holstein cows ($n = 25$) with regular oestrous cycles (Díaz *et al.*, 2015). Ovarian activity was synchronized starting with the procedure commonly referred to as 'G6G' (Bello *et al.*, 2006), with some modifications (Fig. 1; Díaz *et al.*, 2015). Briefly, the synchronization protocol consisted of two doses of prostaglandin F2α (PGF2α; 150 μg D + cloprostenol; Enzaprost DC, Biogénesis-Bagó, Garín, Buenos Aires, Argentina) administered 12 h apart on day 0 to induce luteolysis (Hatler *et al.*, 2008), followed by a dose of GnRH (20 μg buserelin acetate; Gonaxal, Biogénesis-Bagó, Argentina) 2 days later to stimulate ovulation of the pre-ovulatory follicles present. Six days after the first dose of GnRH, the cows were given another injection of GnRH. Seven days later, the cows received two doses of PGF2α, 12 h apart, to ensure luteolysis (completion of the modified synchronization protocol). After synchronization, cows were divided into five groups: control (C; $n = 5$), cows receiving no additional hormonal treatment; P0 group ($n = 5$), cows treated with progesterone from day 1 after the final doses of PGF2α until sampling on the expected day of ovulation; P5 group ($n = 5$), cows with 5 days of follicular persistence after the expected day of ovulation; P10 group ($n = 5$), cows with 10 days of follicular persistence after the expected day of ovulation and; P15 group ($n = 5$), cows with 15 days of follicular persistence after the expected day of ovulation (Fig. 1). To obtain the persistence groups, cows were given a low dose of progesterone by inserting an intravaginal progesterone-releasing device (750 mg of micronized progesterone; Pro-Ciclar P4-Zoovet®; Santa Fe, Argentina) one day after the first PGF2α injection of the Ovsynch protocol (Bello *et al.*, 2006). In the last two groups (P10 and P15), a new intravaginal progesterone-releasing device was inserted 1 day before removal of the first one in order to maintain a more consistent concentration of progesterone throughout the treatment period. In group P15, a third intravaginal progesterone-releasing device was inserted on day 11 of persistence, 1 day before removal of the second one.

Collection and Preparation of Tissues

Bilateral ovariectomy was performed 2 days after completion of the synchronization protocol in control

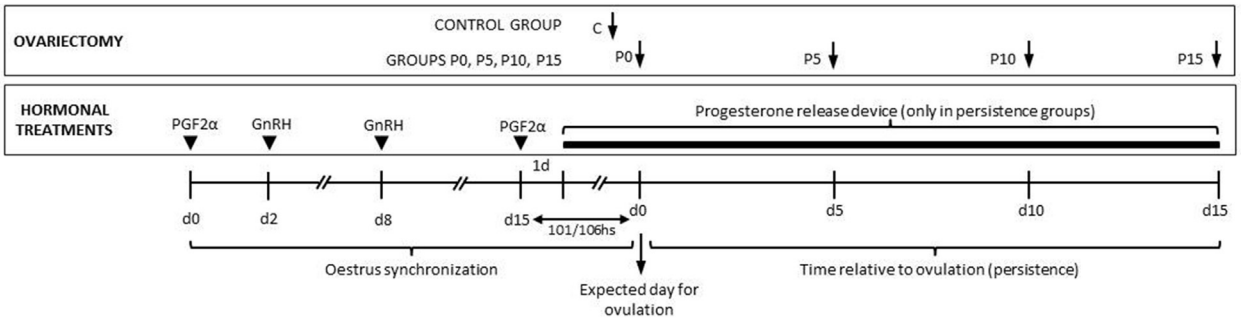

Fig. 1. Experimental design for the induction of follicular persistence. Holstein cows were synchronized and received an intravaginal progesterone-releasing device to induce follicular persistence. Ovariectomy was performed at proestrus (controls) and after 0 (expected day for ovulation), 5, 10 and 15 days of follicular persistence.

cows, on the expected day of ovulation in group P0 and on days 5, 10 and 15 of follicular persistence in groups P5, P10 and P15, respectively (Fig. 1) (Marelli *et al.*, 2014; Díaz *et al.*, 2015). Briefly, animals were sedated with 150 mg of ketamine hydrochloride (10% ketamine, Alfasan, Santa Fe, Argentina) and low epidural anaesthesia was performed with 100 mg of lidocaine hydrochloride (2% lidocaine, Vetue, Santa Fe, Argentina). The perineal area and the vaginal canal were cleaned and disinfected with a solution of 5% chloroxylenol. Then, using a retractor, the vaginal vault was placed under tension and moved away from the rectum (cranio-ventral direction) to avoid contact. A hidden dismountable blade scalpel was introduced and a 5 cm longitudinal incision was performed on the mucosa of the vaginal vault, beginning the incision from cranial to caudal at about 7 cm from cervix. The next step was to introduce three fingers into the pelvic cavity, locate and retract the uterus together with the ovaries into the vagina. At this site, the blood vessels that supply both ovaries were ligated by transfixion and then the ovaries were removed.

Blood samples were obtained daily throughout the experiment to test hormone levels (Díaz *et al.*, 2015). After ovariectomy, FF from dominant pre-ovulatory follicles of the control group and persistent follicles from each persistence group, was aspirated and stored at −80°C until use. Tissue fractions of ovaries with persistent follicles and pre-ovulatory control follicles were frozen immediately in liquid nitrogen and then stored at −80°C until use. For immunohistochemistry (IHC), the ovaries were fixed in formaldehyde 4% at room temperature for 8−12 h. The fixed tissues were washed in phosphate buffered saline (PBS), dehydrated and embedded in paraffin wax. Sections (5 μm) were mounted on slides previously treated with 3-aminopropyltriethoxysilane (Sigma−Aldrich, St. Louis, Missouri, USA) and stained with haematoxylin and eosin (HE) for preliminary observation of the ovarian structures (Díaz *et al.*, 2015). Considering the histological characteristics, follicles were classified into primary, secondary or tertiary (all groups) (Priedkalns, 1998) and persistent follicles (follicular persistence groups) (Díaz *et al.*, 2015).

Follicular health status was confirmed by morphology and hormonal concentrations in FF (Díaz *et al.*, 2015).

Immunohistochemistry

Protein expression of IGFR1 and IGFBP4 was detected using polyclonal antibodies (Table 1). The streptavidin−biotin−immunoperoxidase method (CytoScan™ HRP detection system, Cell Marque, Rocklin, California, USA) was carried out as described previously (Rodríguez *et al.*, 2013, 2015). Briefly, after dewaxing, the IGFR1 antigen was retrieved by incubating the sections in 0.01 M citrate buffer (pH 6.0) in a microwave oven at 800 W. Detection of IGFBP4 was carried out without recovery of the antigen. Endogenous peroxidase activity was inhibited with H_2O_2 3% in methanol, and non-specific binding was blocked with 10% (v/v) normal goat serum. Slides were incubated with polyclonal rabbit anti-IGFR1 (Table 1) for 18 h at 4°C and polyclonal rabbit anti-IGFBP4 (Table 1) for 18 h at 25°C. Slides were then incubated for 30 min at room temperature with biotinylated secondary antibody (Table 1). For antigen visualization, 3, 3′diaminobenzidine (DAB) was used as the chromogen. To verify specificity of labelling, adjacent control sections were subjected to the same immunohistochemical method, replacing primary antibodies with rabbit non-immune sera. The specificity of the secondary antibody was tested by incubation with anti-human oestrogen receptor alpha (polyclonal antibody, Cell Marque), a primary antibody with

Table 1
Reagents used for immunohistochemistry and western blotting

Antibodies	*Clone/source*	*Dilution*	
		IHC	*WB*
Primary antibodies			
Anti-PAPP-A	SC-50518. Rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, California, USA)	–	1 in 400
Anti-IGFR1	SC-712. Rabbit polyclonal (Santa Cruz Biotechnology)	1 in 100	
Anti-IGFBP4	Polyclonal antiserum (Novozymes GroPep Ltd., Thebarton, Adelaide, South Australia)	1 in 150	1 in 700
Secondary antibodies			
Goat polyclonal	65-6140 (Zymed, San Francisco, California, USA)	6 μg/ml	–
HRP-anti rabbit IgG	SC-2004 (Santa Cruz Biotechnology)	–	1 in 7,500

IHC, immunohistochemistry; WB, western blotting.

no reactivity with bovine antigens. To exclude the possibility of non-suppressed endogenous peroxidase activity, some sections were incubated with DAB alone. The specificity of the primary antibodies used in this study was evaluated previously by western blot (Rodríguez *et al.*, 2013, 2015).

Western Blotting

The amount of IGFBP4 and PAPP-A secreted was determined by western blot in FF of persistent follicles of groups P0, P5, P10 and P15 and pre-ovulatory follicles of the control group.

Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) 8% and 15% (w/v) acrylamide–polyacrylamide for PAPP-A and IGFBP4 detection, respectively, and then transferred onto nitrocellulose membranes (Hybond ECL nitrocellulose membrane, GE Healthcare, Buckinghamshire, UK). The membranes were blocked with Tris buffered saline (TBS) containing 0.05% (v/v) Tween20 (Sigma–Aldrich) and 2% (w/v) non-fat milk, and then incubated overnight at 4°C with specific primary antibodies (Table 1). Bound antibodies were detected using anti-rabbit IgG peroxidase antibody (Table 1). The immunopositive bands were detected by chemiluminescence, using the ECL-plus system (GE Healthcare) on hyperfilm-ECL film (GE Healthcare).

Hormone Assays

For radioimmunoassay of IGF1, 15 μl of FF or IGF1 standards were subjected to the acid–ethanol cryoprecipitation method, which eliminates possible residual IGFBPs (Breier *et al.*, 1991; Lacau-Mengido *et al.*, 2000; Ortega *et al.*, 2008). IGF was determined using an antibody (UB2-495) provided by Drs. L. Underwood and J. J. Van Wyk, and distributed by the Hormone Distribution Programme of the NIDDK. Recombinant human IGF1 (rhIGF1, Chiron Corp., Emeryville, California, USA) was used as radioligand and unlabelled ligand. The assay sensitivity was 6 pg/tube.

Image Analysis

Images were analyzed using the Image Pro-Plus 3.0 system (Media Cybernetics, Silver Spring, Maryland, USA). Images were digitized using a colour video camera (Motic 2000; Motic China Group, Xiamen, China) mounted on the top of a conventional light microscope (Olympus BH-2, Olympus, Co., Tokyo, Japan), as described previously (Ortega *et al.*, 2009, 2010). The methodological details of image analysis as a valid method for quantification have been described previously (Ranefall *et al.*, 1998; Ortega *et al.*, 2009).

The positive controls were used as interassay controls to maximize the levels of accuracy and

robustness of the method (Ranefall *et al.*, 1998). The slides were scanned left to right from the top and all follicles of the selected categories were analyzed. The image analysis score was calculated separately in each follicular wall layer (i.e. granulosa and theca interna) from at least 50 images of the different categories of follicles from ovaries of all groups. Antigen expression in tissue sections was expressed as a fraction of the labelled area (% of immunopositive area) and was calculated as a percentage of the total area evaluated through colour segmentation analysis, which extracts objects by locating all objects of the specific colour (i.e. brown label). These values were verified and normalized with the positive controls carried across various runs using the same region (verified by image comparison) for calibration. Sections were analysed with the observer blinded to the experimental group. Only one person performed the analysis of each marker so as to maintain the same criteria in the image analyzes.

The main strength of the well-validated imaging approach used in this study is the visualization of the in-situ localization of proteins within cells of interest. This approach has been successfully applied to immunoreactivity in different tissues (Ranefall *et al.*, 1998; Ortega *et al.*, 2009).

For the western blot, the exposed films were scanned at 1,200 dpi (scanner HP Officejet J5, 780; Hewlett−Packard). IGFBP4 and PAPP-A levels were analyzed by densitometry to obtain an integrated optical density (IOD) value, which was compared between FF samples from preovulatory follicles of the control group and persistent follicles of follicular persistence groups (Rey *et al.*, 2010).

Statistical Analysis

The adequate number of images per follicle and the number of follicles per category were confirmed from a sample size calculation that evaluated the number of samples necessary to produce an estimate of the immunoreactivity that would fall within 0.4 units of the real value.

A statistical software package (SPSS 11.0 for Windows, SPSS Inc., Chicago, Illinois, USA) was used to analyze the data. The distribution of data was tested for normality using the Kolmogorov−Smirnov test. Tests of homogeneity of variance between groups were conducted using Levene's test. The statistical significance of differences between groups of data (comparison of groups for each follicular category and within each group along follicular development) was assessed by one-way ANOVA, followed by Duncan's multiple range tests as a multiple comparison test. $P < 0.05$ was considered significant. The Student's t-test was used to evaluate levels of changes in protein expression between control tertiary and persistent follicles of each group (P0, P5, P10 and P15). The reference structures for comparison with persistent follicles were healthy tertiary follicles, with no signs of atresia and obtained in proestrus. These follicles are considered adequate to perform the comparison since they are selected in the follicular wave and have the hormonal characteristics of the follicles intended to ovulate (Berisha *et al.*, 2002; Aerts and Bols, 2010). The results were expressed as mean ± standard deviation (SD).

Results

Protein Expression of IGFR1 and IGFBP4 in Ovarian Samples

IGFR1 and IGFBP4 expression was evaluated in the follicles of the different groups. IGFR1 was detected in granulosa cells (Fig. 2), while IGFBP4 was detected in both granulosa and theca cells (Fig. 3). Fig. 4A shows IGFR1 expression for different follicular categories within each group. In the control group, IGFR1 expression was higher in tertiary follicles than in primary and secondary follicles (Fig. 4A). In groups P0, P5, P10 and P15, IGFR1 expression was higher in tertiary and persistent follicles than in primary and secondary follicles. IGFR1 expression was higher in the granulosa of persistent follicles of P0, P5, P10 and P15 than in control tertiary follicles considered as reference structures ($P < 0.05$).

The comparison between groups showed that IGFR1 expression in primary and secondary follicles was similar. In addition, IGFR1 expression was higher in tertiary follicles of groups P0, P5 and P15 than in those of the control group (Fig. 4B). No differences were detected between the persistent follicles of the different treated groups ($P > 0.05$).

Fig. 5A shows IGFBP4 expression in granulosa cells for different follicular categories within each group. In the control group, IGFBP4 expression was lower in tertiary follicles than in primary follicles. A similar pattern was found for the P0 group, where IGFBP4 expression was lower in tertiary follicles than in primary and secondary follicles, without differences with persistent follicles. In group P5, IGFBP4 expression was higher in tertiary follicles than in primary follicles, without differences with persistent follicles. No differences were observed for groups P10 and P15 (Fig. 5A). In granulosa cells of persistent follicles of groups P0, P5, P10 and P15, IGFBP4 expression was higher than in control tertiary follicles ($P < 0.05$) (Fig. 5A).

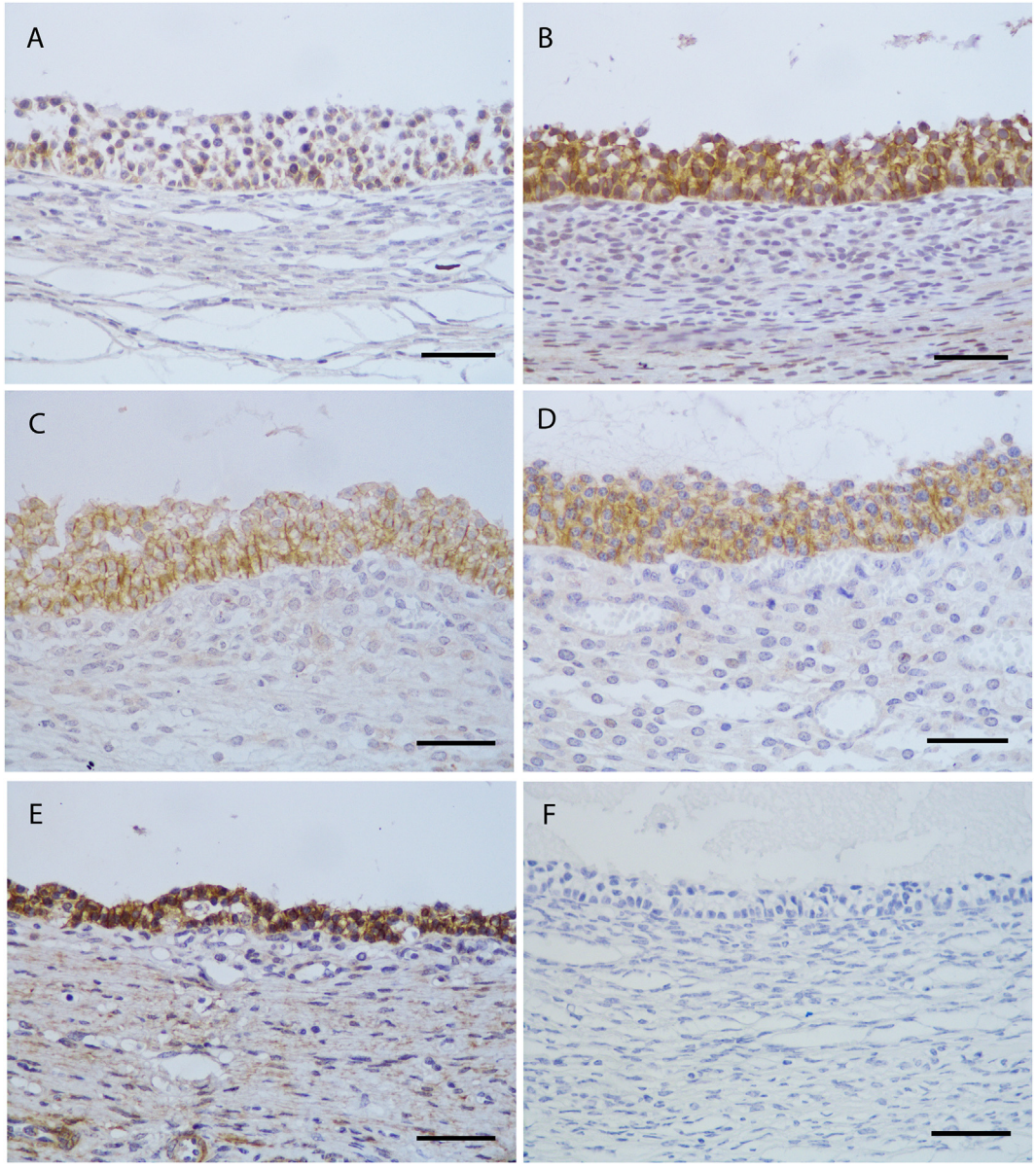

Fig. 2. Representative images of IGFR1 protein localization in follicles. The immunoreactivity was intense in granulosa cells, and no expression was detected in theca cells in the follicular structures analyzed. Images represent (A) a control tertiary follicle, (B) a follicle that showed lower IGFR1 expression than follicle persisting for 0 days, (C) a follicle persisting for 5 days, (D) a follicle persisting for 10 days (D), and (E) a follicle persisting for 15 days. No labelling was observed when the primary antibody was replaced with normal rabbit serum (negative control, F). Diffuse immunolabelling corresponds to non-specific background. IHC. Bars, 25 μm.

IGFBP4 expression in granulosa cells was also compared between groups for each follicular category (Fig. 5B). In primary follicles, IGFBP4 expression was lower in the control group than in groups P0 and P10. In secondary follicles, IGFBP4 expression was lower in the control group than in groups P0, P5, P10 and P15. In tertiary follicles, IGFBP4 expression was higher in groups P5, P10 and P15 than in the control and P0 groups. No differences were detected between persistent follicles from the groups evaluated. No differences were found in IGFBP4 expression of theca cells in any of the follicular categories analyzed (Fig. 5C).

Protein Expression of PAPP-A and IGFBP4 in Follicular Fluid

PAPP-A levels in FF from tertiary follicles of the control group and persistent follicles of groups P0, P5, P10 and P15 were similar (Fig. 6). However, a tendency to an increase in persistent follicles of group P10 was observed in comparison with group P0 ($P = 0.057$).

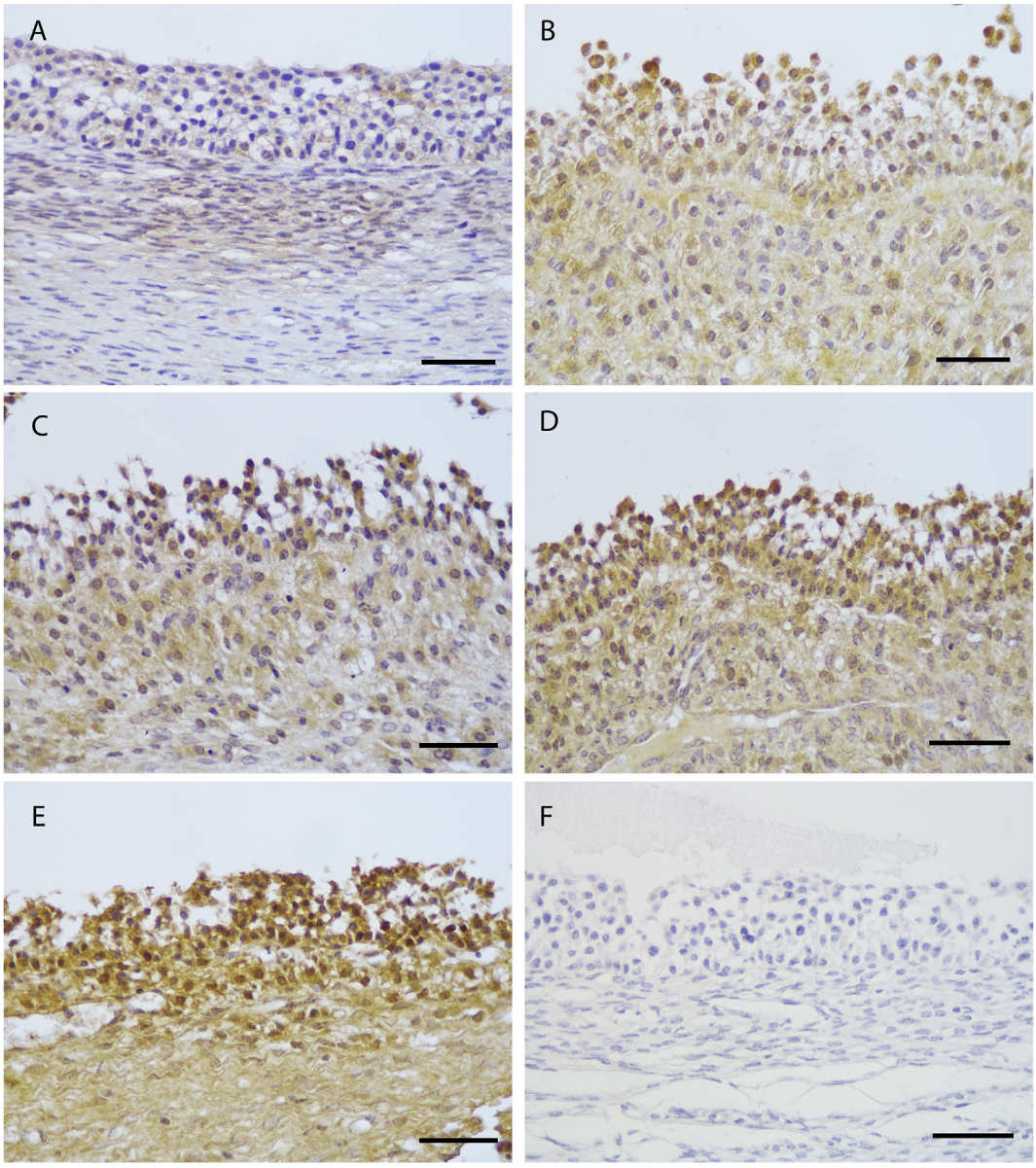

Fig. 3. Representative images of IGFBP4 protein localization in follicles. The immunoreactivity was intense in granulosa cells, and no expression was detected in theca cells in the follicular structures analyzed. Images represent (A) a control tertiary follicle, (B) a follicle persisting for 0 days, (C) a follicle persisting for 5 days, (D) a follicle persisting for 10 days, and (E) a follicle persisting for 15 days. No labelling was observed when the primary antibody was replaced with normal rabbit serum (negative control, F). Diffuse immunolabelling corresponds to non-specific background. IHC. Bars, 25 μm.

IGFBP4 was identified in its glycosylated (29 kDa) and non-glycosylated forms (24 kDa) by western blot. Similar levels of both forms were detected in FF from control tertiary follicles and persistent follicles of groups P0, P5, P10 and P15 (Fig. 7).

Concentrations of IGF1 in Follicular Fluid

Similar concentrations of IGF1 were detected in FF from tertiary follicles from the control group and persistent follicles from the different treated groups (Table 2).

Discussion

Our previous studies on the IGF system in bovine follicular cysts were carried out using slaughterhouse material or cows with already established COD, where the reproductive history of the cows and persistence of cysts were unknown (Rey *et al.*, 2010; Rodríguez *et al.*, 2011), or in experimental models

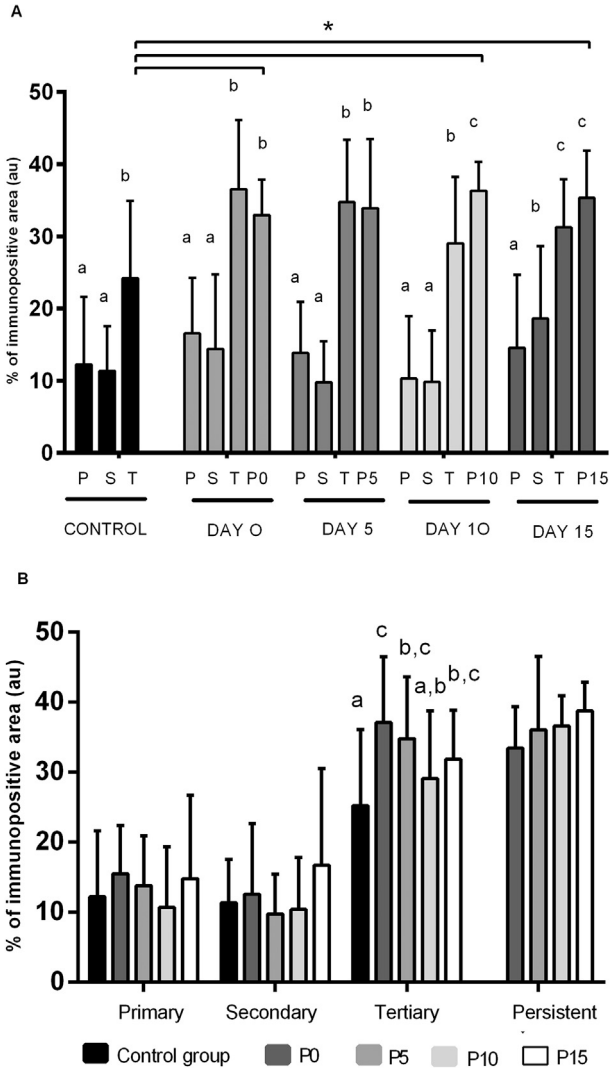

Fig. 4. Immunohistochemical labelling of IGFR1 in granulosa cells (A, B) of developing follicles (primary [P], secondary [S] and tertiary [T] follicles) of the control, P0 (0 days of follicular persistence), P5 (5 days of follicular persistence), P10 (10 days of follicular persistence) and P15 (15 days of follicular persistence) groups. (A) IGFR1 expression for different follicular categories within each group. (B) Comparison of IGFR1 expression between experimental groups. Bars represent the percentage of immunolabelled areas as the mean ± standard deviation (SD). Asterisk and different letters denote significant differences ($P < 0.05$). au: arbitrary units.

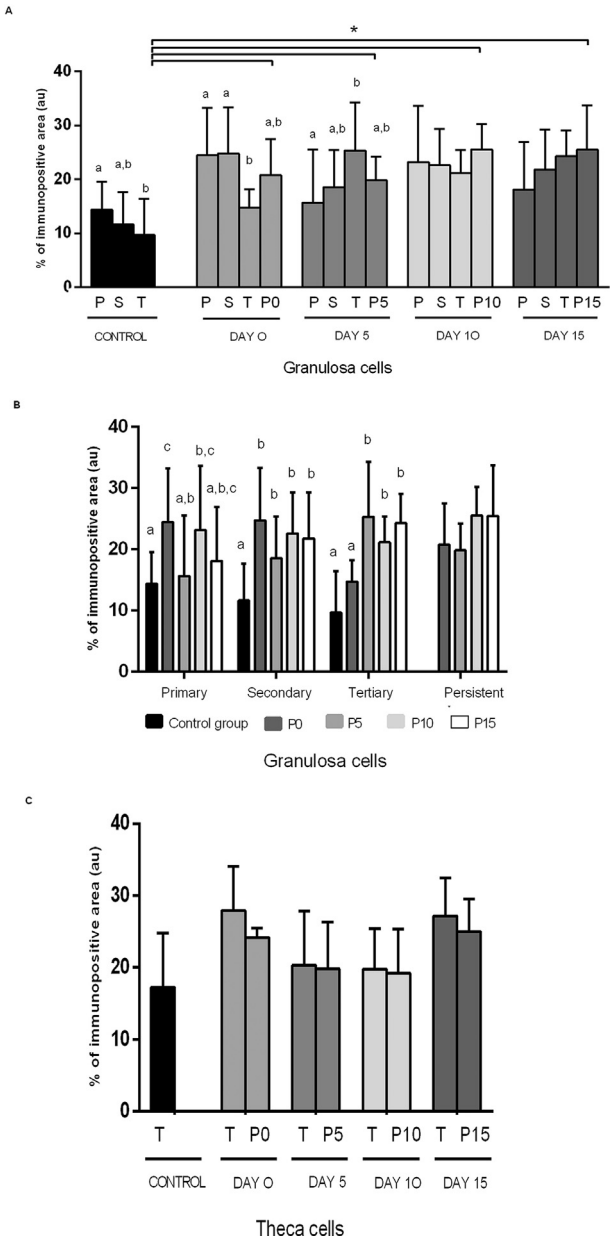

Fig. 5. Percentage of immunopositive areas of IGFBP4 labelling of granulosa (A, B) and theca (C) cells evaluated in developing follicles (primary [P], secondary [S] and tertiary [T] follicles) of the control, P0 (0 days of follicular persistence), P5 (5 days of follicular persistence), P10 (10 days of follicular persistence) and P15 (15 days of follicular persistence) groups. (A) IGFR1 expression for different follicular categories within each group. (B) Comparison of IGFR1 expression between experimental groups. Bars represent the mean ± standard deviation (SD). Asterisk and different letters denote significant differences ($P < 0.05$). au: arbitrary units.

that did not evaluate what happens during the early stages of development of the disease (Rodríguez *et al.*, 2013, 2015). In the present study, an experimental model of follicular persistence was used to evaluate the main components of the IGF system in the early stages of development of COD. The experimental model used was optimized previously and proven to be suitable to study the early stages of cyst development in cattle (Díaz *et al.*, 2015, 2016).

In accordance with previous studies, IGFR1 expression was detected in granulosa cells of follicles at all stages of development, but not in theca cells (el-Roeiy *et al.*, 1993, Lucy, 2008; Silva *et al.*, 2009;

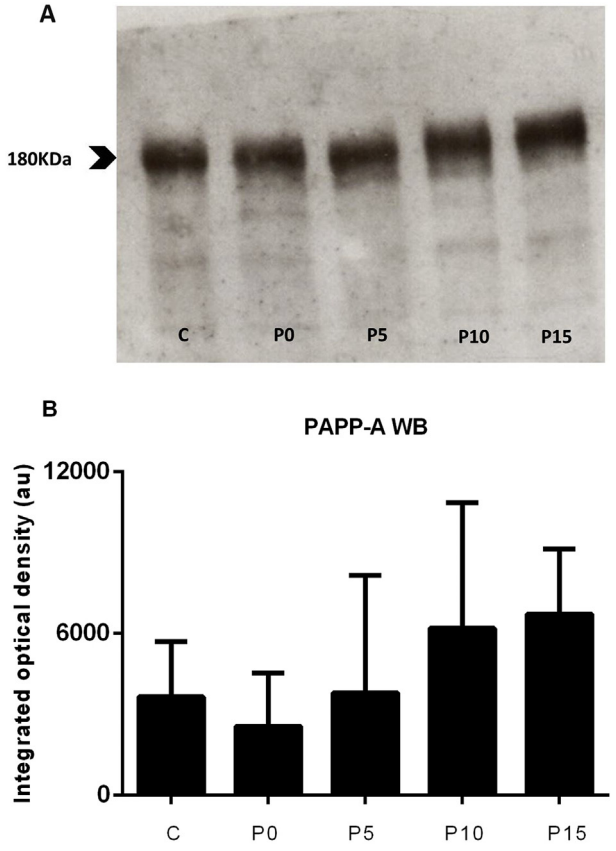

Fig. 6. Representative image of detection of PAPP-A protein secreted to the bovine follicular fluid by western blot of follicles from the control (C), P0 (0 days of follicular persistence), P5 (5 days of follicular persistence), P10 (10 days of follicular persistence) and P15 (15 days of follicular persistence) groups. (B) Levels of secreted PAPP-A in follicular fluid of follicles from the control (C), P0, P5, P10 and P15 groups. Bars represent the mean ± standard deviation (SD). au: arbitrary units.

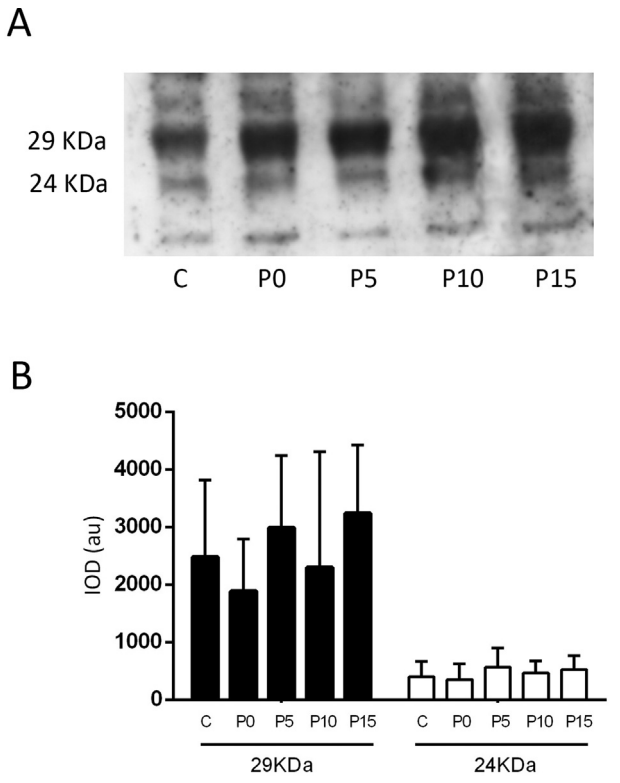

Fig. 7. (A) Representative image of detection of IGFBP4 protein in bovine follicular fluid by western blot of follicles from the control (C), P0 (0 days of follicular persistence), P5 (5 days of follicular persistence), P10 (10 days of follicular persistence) and P15 (15 days of follicular persistence) groups. (B) Levels of glycosylated IGFBP4 (29 KDa) and non-glycosylated IGFBP4 (24 KDa) in follicular fluid of follicles from the control (C), P0, P5, P10 and P15 groups. Bars represent the mean ± standard deviation (SD) au: arbitrary units.

Table 2
Concentration of IGF1 in follicular fluid of control heifers with normal preovulatory follicles and follicles from 0 (P0), 5 (P5), 10 (P10) and 15 (P15) days of follicular persistence

Groups	*IGF1 (ng/ml)*
Control	126 ± 76
P0	107 ± 80
P5	130 ± 62
P10	156 ± 63
P15	184 ± 99

Rodríguez *et al.*, 2015). Unlike that observed by other authors (Sanchez *et al.*, 2014), in this study, IGFR1 expression was higher in tertiary follicles than in primary and secondary follicles in all the groups evaluated. Furthermore, in accordance with previous studies of our group (Rodríguez *et al.*, 2015), IGFR1 expression was higher in persistent follicles than in control tertiary follicles.

Some authors have postulated that the IGFR1 activity can be ligand-dependent, while others suggest that IGFR1 is activated 17b-oestradiol (E2) via a mechanism that does not involve binding of IGF1 or IGF2 to the receptor (Kamanga-Sollo *et al.*, 2013; Kavran *et al.*, 2014). Our previous studies also showed higher levels of IGFR1 in cysts, and we postulated a possible stimulation by constant levels of E2 in these structures (Amweg *et al.*, 2013; Rodríguez *et al.*, 2015). Similarly, the higher levels of IGFR1 detected in this study in persistent follicles since day 0 (expected time of ovulation) could be due to the high levels of E2 in the serum of animals with follicular persistence (Díaz *et al.*, 2015), besides the constant concentrations of IGF1 shown. Therefore, high levels of IGFR1 throughout follicular persistence would be able to maintain the response to IGF1, such as

steroidogenesis (Ortega *et al.*, 2008; Amweg *et al.*, 2013; Rodríguez *et al.*, 2015).

In the present study, we detected IGFBP4 protein in FF and expression in granulosa and theca cells, in accordance with previous results (Armstrong *et al.*, 1998; Roberts and Echternkamp, 2003; Rodríguez *et al.*, 2013). All of the follicular structures of the persistence groups showed higher IGFBP4 expression than in the control group. In granulosa cells, IGFBP4 levels were higher in persistent follicles of groups P0, P5, P10 and P15 than in tertiary follicles of the control group. It has been suggested that LH is involved in a direct way, increasing IGFBP4 production and degradation by also increasing PAPP-A expression (Hsu and Hammond, 1987; Hastie and Haresign, 2010). We have previously shown that the basal concentration of LH in the follicular phase is lower in control animals than in cows with progesterone-induced follicular persistence (Díaz *et al.*, 2015). Studies in ruminants have shown an association between the reduced IGFBP4 levels in FF during follicular growth and those in spontaneously arising cysts, which could be due to a regulation of mRNA synthesis or a high level of degradation by high levels of PAPP-A (Monget *et al.*, 1993, 2002; Spicer and Echternkamp, 1995; Stewart *et al.*, 1996; Rodríguez *et al.*, 2013; Meyerholz *et al.*, 2015). Therefore, the higher IGFBP4 expression detected in persistent follicles could be due to an attempt to balance the levels of IGFBP in the follicles because of degradation by PAPP-A or regulation of synthesis by LH. Furthermore, in the present study, we analyzed the levels of IGFBP4 in FF, where IGF availability is effectively regulated. We detected two bands corresponding to the non-glycosylated (24 KDa) and glycosylated (29 KDa) forms. It should be noted that previous reports have shown that glycosylation does not affect the affinity of IGFBP4 for IGF1 (Clemmons, 2011). IGFBP4 is cleaved in physiological fluids; as a consequence, 16 KDa and 14 KDa fragments are generated. These proteolytic fragments have very low affinity for IGF1 (Clemmons, 2011). Therefore, the IGFBP4 detected here by western blot would be the form available to bind IGF1.

Previous studies of bovine FF have documented changes in IGFBP levels during follicle growth and development (de la Sota *et al.*, 1996; Stewart *et al.*, 1996). Active IGFBP4 levels were lower in large tertiary follicles than in small follicles, without differences between large follicles and spontaneously arising cysts (Rodríguez *et al.*, 2013). Austin *et al.* (2001) also detected decreased levels of IGFBP4 in healthy dominant follicles. In this study, IGFBP4 levels in FF from follicles at different times of persistence and those from control tertiary follicles were similar. As IGFBP4 expression in granulosa cells was different between groups, the secreted IGFBP4 levels found in FF could be due either to a differential regulation of IGFBP secretion or to changes in the selective uptake of IGFBPs from the circulation in persistent follicles. Considering that IGFBP4 acts as an inhibitor of IGF action, these results suggest that IGFBP4 might be protecting cells from the action of IGF1 by sequestering it (Clemmons, 2011; Ortega *et al.*, 2008).

The essential mechanism for the release of IGFs at the site of target cells is an enhanced protease activity of the enzymes that degrade IGFBPs (Meyerholz *et al.*, 2015). PAPP-A is the main IGFBP protease regulating the levels of free IGF1 in the bovine dominant follicle (Mazerbourg *et al.*, 2001; Spicer *et al.*, 2004; Sanchez *et al.*, 2014). Some studies have shown that PAPP-A tends to increase as the follicle develops and that PAPP-A is able to keep lower levels of IGFBP4 in FF (Mazerbourg *et al.*, 2001; Fortune *et al.*, 2004; Spicer *et al.*, 2004; Santiago *et al.*, 2005; Rodríguez *et al.*, 2013, 2015) and, accordingly, higher availability of IGF1 for follicular growth (Spicer *et al.*, 1993, 1996, 2004; Fortune *et al.*, 2004; Rodríguez *et al.*, 2013, 2015). Similar to the findings of other authors, we found PAPP-A protein in FF, most likely secreted by the granulosa cells (Mazerbourg *et al.*, 2001; Monget *et al.*, 2003; Rivera and Fortune, 2003; Rodríguez *et al.*, 2015). In accordance with previous studies, where no differences were found between cysts and control tertiary follicles (Rodríguez *et al.*, 2015), in this study, we found similar PAPP-A levels secreted to FF in persistent follicles and control follicles.

Cleavage of IGFBP4 by PAPP-A requires the binding of IGF1 or IGF2 to this IGFBP (Qin *et al.*, 2000; Laursen *et al.*, 2001; Oxvig, 2015). PAPP-A activity towards the IGFBP4/IGF complex has high kinetic efficiency, while cleavage of IGFBP4 in the absence of IGF is negligible (Gyrup and Oxvig, 2007). Cleavage occurs in close proximity to IGFR1, increasing the chance that released IGF will stimulate the receptor (Laursen *et al.*, 2002). Moreover, dominant follicles have higher PAPP-A synthesis and subsequently have lower concentrations of IGFBP4 and IGFBP5 in the FF (Mazerbourg *et al.*, 2001; Rivera and Fortune, 2001; Fortune *et al.*, 2004). The PAPP-A levels detected in FF could therefore be due to regulated secretion of this enzyme or to synthesis and release from other tissue structures such as kidney, bone or corpus luteum, acting in a paracrine or endocrine fashion (Mazerbourg *et al.*, 2001; Spicer, 2004; Conover, 2012). Subsequently, the IGFBP4

synthesized by granulosa cells would be released and bind to IGF1 in the FF. During the initial stages of persistence, the constant levels of PAPP-A detected would be in agreement with IGFBP4 levels that do not alter the IGF1 concentrations detected in persistent follicles.

IGF1 plays important roles in the regulation of cellular metabolism and growth of vertebrates. It also acts as a potent mitogen, regulating the balance of cellular proliferation, differentiation and survival (Giudice, 1992; Singh and Rubin, 1993; Cantero *et al*., 2007).

The bioavailability of IGFs is the result of a relationship between IGFBPs and proteases (Mazerbourg *et al*., 2001; Boldt and Conover, 2007; Ning *et al*., 2008). It has been suggested that IGF1 is involved not only in the pathogenesis, but also in the maintenance of COD in cattle (Zulu *et al*., 2002; Probo *et al*., 2011). Previous studies have found low levels of IGF1 in FF of animals with spontaneously arising or adrenocorticotropic hormone-induced cysts (Beam and Buttler, 1997, 1998; Zulu *et al*., 2002; Ortega *et al*., 2008). In this study, the concentrations of IGF1 in FF were similar in all stages of persistence and in control animals. These results suggest a bioavailability resulting from the cleavage of IGFBP4 through PAPP-A. On the other hand, it has been shown that E2 induces IGF1 synthesis (Hsu and Hammond, 1987; Spicer and Chamberlain, 2000). In previous studies, we determined lower concentration of E2 in FF from follicles persisting for 15 days than in control pre-ovulatory follicles and follicles persisting for 5 and 10 days (Díaz *et al*., 2015). Therefore, we could infer that, in spontaneous COD, E2 would promote IGF1 synthesis during the early stages of the disease, and that once COD has developed, this synthesis would not be maintained, leading to low IGF1 concentrations (Ortega *et al*., 2008; Rodríguez *et al*., 2013). Considering these results and the fact that IGF1 participates in proliferation, modulation of apoptosis and steroidogenesis, we suggest that IGF1 is responsible for maintaining cysts (Ortega *et al*., 2008; Salvetti *et al*., 2010; Amweg *et al*., 2013). Finally, we could hypothesize that the similar levels of PAPP-A detected in this study might be necessary to increase the degradation of the high levels of IGFBP4 that is being synthesized in granulosa cells and released to FF of the persistent follicles at the initial stages of the persistence. Therefore, in persistent follicles, similar concentrations of IGF1 would be free and available to bind to high levels of IGFR1. Consequently, IGF1 can undertake its biological functions, such as steroidogenesis and cellular proliferation and survival, and so contribute to follicular persistence and the maintenance of cysts.

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Conflict of Interest Statement

The authors declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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