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Interaction between IGF1 and IGFBPs in bovine cystic ovarian disease

Short title: IGF1 and IGFBPs in bovine cystic ovaries

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ABSTRACT

Cystic ovarian disease (COD) is one of the main factors responsible for reproductive disorders in cattle. Although the pathogenesis and mechanism of cyst formation are not fully understood, it has been proposed that the IGF system could play an essential role, as it is a key intraovarian regulator. The aim of the present study was to determine whether the altered levels in IGF1 detected in bovines with COD are associated with changes at mRNA level or with differential modulation by IGFBPs. The mRNA levels of the IGF components studied were analyzed by real time PCR and in situ hybridization, and IGFBP expression and activity were assayed by immunohistochemistry and ligand blot respectively. Results showed a decreased IGF1 mRNA level due to a lower granulosa cell gene expression in cystic follicles ($P < 0.05$). Results also showed variations in IGFBP expression in the intraovarian cellular compartment and concentration in follicular fluid, and suggest that IGFBP3 is a key regulator of intrafollicular IGF1 in animals with COD.

Keywords: cattle, cystic ovarian disease, insulin-like growth factor, ovaries

INTRODUCTION

Ovarian follicular cysts in cattle are characterized by large anovular structures that persist in the ovary for approximately 10 days in the absence of a corpus luteum, with interruption of the normal estrous cycle (Silvia et al., 2002; Vanholder, 2006). The incidence of this disease has been estimated to occur in up to 30% of dairy cows, and the number of cases has increased in recent years (Garverick, 1997; Silvia et al., 2005; Vanholder et al., 2006), probably due to the selection of high-yielding animals.

The pathogenesis and mechanism of cyst formation are not fully understood. One of the main difficulties in investigating ovarian cysts in bovines is that there are different factors contributing to cyst formation (Vanholder et al., 2006). However, a hypothalamic unresponsiveness to an estradiol surge appears to be one of the underlying causes of follicular cyst formation in dairy cows (Gümen and Wiltbank, 2002). It is believed that an altered feedback mechanism of estrogens in the hypothalamic-pituitary axis can result in an aberrant GnRH and LH release and cyst formation, where growth factors play an active role.

The insulin-like growth factor (IGF) system is a key intraovarian regulator and the successful development of a healthy oocyte and appropriate granulosa and theca cell steroidogenesis in the estrous cycle depend on multiple factors, including the proper function of this system (Jones and Clemmons, 1995; Spicer, 2004). Disruption of even one component of this system can lead to abnormal follicular development and function, and compromise the reproductive capacity by contributing to the development of follicular cysts (Zulu et al., 2002; Ortega et al., 2008; Rey et al., 2010; Probo et al., 2011).

The bioavailability of both IGF1 and IGF2 in the follicle is intimately influenced by the relative proportions of IGF-binding proteins (IGFBPs) (Firth and Baxter, 2002; Rey et al., 2010;

Rodríguez et al., 2011) and by the different affinities between the ligands and individual IGFBPs (Jones and Clemmons, 1995; Rey et al., 2010). Six IGFBPs (IGFBP1 to 6) have been described to bind IGF1 and IGF2, prolong their half-lives, and block their action in most situations (Rechler, 1993; Firth and Baxter, 2002). On the other hand, evidence exists for proteolytic enzymes that degrade and inactivate IGFBPs during follicular development in mammals. These protease-induced decreases in IGFBPs likely cause increased levels of free IGFs, which stimulate steroidogenesis and mitogenesis (Spicer, 2004). Therefore, IGFs are regulated at various levels, with a constantly shifting contribution of endocrine, autocrine and paracrine factors.

Given the role of the IGF system in the ovarian function, we hypothesized that an imbalance in this system may result in ovarian alterations such as cystic ovarian disease (COD). Therefore, the aim of the present study was to determine whether the altered levels of IGF1 previously detected in cattle with COD (Ortega et al., 2008) are associated with changes at mRNA level or with a differential modulation by IGFBPs.

MATERIALS AND METHODS

Induced cystic follicles and controls

All procedures were carried out according to the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science Societies, 1999) and the protocol was approved by the Ethics and Safety Committee of the Faculty of Veterinary Sciences (Universidad Nacional del Litoral, Argentina). Ten nulliparous Argentinean Holstein heifers (18 to 24 months old; 400 to 450 kg body weight; maintained under standard husbandry conditions) with regular estrous cycles according to prior detection of estrus, rectal palpation and ultrasonography, were used. Their estrous cycles were

synchronized using the Ovsynch protocol as described previously (Ortega et al, 2008; Rodríguez et al., 2011). The day on which estrous behavior was first detected was confirmed by rectal examination and ultrasonography, and designated Day 0 of the cycle (Gümen et al., 2003). Beginning on Day 15 of a synchronized estrous cycle, five heifers received subcutaneous injections of tetracosactrin hexaacetate (1 mg/ml/animal; Synacthen Depot, Novartis, Basel, Switzerland), a synthetic polypeptide with ACTH activity, every 12 h for 7 days (Ortega et al., 2008). Five control animals received saline (1 ml) (Dobson et al., 2000; Ortega et al., 2008). Ultrasonographic ovarian examinations were performed in all animals, as previously described, using a real-time, B-mode scanner equipped with a 7.5 MHz, linear-array, transrectal transducer (Aloka, SSD 500; Wallingford, CT, USA) (Sirois and Fortune, 1988; Rodríguez et al., 2011). Daily ovarian ultrasonography was performed throughout one complete estrous cycle (21 days in the control group) and from Day 14 (Day 0 = day of estrus) until Day 48 (treated group). Cysts detected by ultrasonography were defined as any follicular structure equal to or greater than 20 mm in diameter that was present for 10 days without forming a corpus luteum (CL) (Dobson et al., 2000; Silvia et al., 2002). The first day of cyst formation was identified retrospectively, 10 days before the daily detection of a follicle equal to or greater than 20 mm in diameter. The ovaries were removed 10 days later, for the identification of follicular cysts, by flank laparotomy. Control heifers were ovariectomized, to obtain normal growing follicles (approximately Day 18), when the dominant follicle reached a diameter greater than 10 mm. During dissection of the ovaries, the follicular diameter was measured using callipers and follicular fluid from each follicle was aspirated and stored separately at -20°C. Tertiary (antral) follicles dissected from normally cycling ovaries were classified as small (< 5 mm), medium-sized (5–10 mm) or large (> 10 mm) (Parrott and Skinner, 1998). Blood samples were obtained

immediately before ovarian excision and centrifuged at 1000 x g for 30 min. The serum collected was stored at -20 °C until hormonal analysis (parallel studies Ortega et al., 2008; Rey et al., 2010). Luteinization of cystic follicles was discarded by means of hormonal analysis. Follicular fluid was aspirated from both the preovulatory follicles of control animals and induced cystic follicles and then frozen until analysis. The ovaries were fixed in 4 % neutral buffered formalin.

Spontaneous cystic ovaries

Ovaries with spontaneous cystic follicles were obtained from dairy cows by flank laparotomy. Eight pluriparous (mean 3.1 ± 1.5 lactations, range 2-7), high-yielding (mean 25.12 ± 6.23 kg of milk per day at diagnosis) Argentinean Holstein cows affected by COD were previously monitored by ultrasonography, and when a follicle ≥ 20 mm in diameter persisting for 10 days in the absence of a functional CL was detected, animals were ovariectomized. Cystic fluids from animals with COD were aspirated using a probe of the real-time, B-mode scanner equipped with a 7.5 MHz, linear-array, transrectal transducer (Aloka, SSD 500; Wallingford, CT, USA) before ovarian excision. After excision, samples were collected, refrigerated on ice and immediately transported to the laboratory. Follicular cysts were evaluated grossly, microscopically and by hormone analysis. Tissue fractions of ovaries from cystic follicles were immediately frozen at -80 °C until use in gene expression assays. Additional sections of ovarian tissue were fixed in 4 % neutral buffered formalin.

Tissue preparation

For light microscopy, the fixed tissues were dehydrated and embedded in paraffin wax.

Sections (4 μm in thickness) were mounted on slides previously treated with 3-aminopropyltriethoxysilane (Sigma-Aldrich, St. Louis, MO, USA) and assigned for staining with hematoxylin and eosin for preliminary observations of all ovarian structures (Salveti et al., 2004) or for use in immunohistochemistry (IHC) or in situ hybridization (ISH). Follicles were classified into primary, secondary, tertiary and cystic follicles (Silvia et al., 2002).

RNA extraction

Total RNA was isolated from the follicular wall of different sized tertiary control follicles and cysts, after treatment with Trizol LS reagent (Invitrogen, Life technology, CA, USA), according to the manufacturer's instructions but with slight modifications (Rey et al., 2010; Rodríguez et al., 2011).

Reverse transcription

To avoid putative genomic DNA contamination, RNA samples were treated with DNase (Invitrogen) according to the manufacturer's instructions. First strand cDNA was synthesized using a master mix (Moloney Murine Leukemia Virus (MMLV) buffer, dithiothreitol (DTT), RNAout, MMLV reverse transcriptase, deoxyribonucleotide triphosphate (dNTP) and random primers (Invitrogen)). The reverse transcription conditions were as described previously (Rodríguez et al., 2011).

Real Time PCR

An optimized real time PCR protocol was used to analyze the mRNA expression of IGF1, IGFBP1, IGFBP4, IGFBP5 and IGFBP6 using SYBR Green I (Invitrogen) technology in LightCycler (Pfaffl et al., 2001). The IGFBP2 and IGFBP3 mRNA had been analyzed in a previous work (Rodríguez et al., 2011).

Transcript levels were measured by relative quantitative real time PCR using a StepOne Real Time PCR System (Applied Biosystems, Life technology, CA, USA). An optimized protocol was used: initial denaturation at 95 °C for 10 min, 36 cycles of denaturation at 95 °C for 15 s and annealing 62 °C (IGF1), 58 °C (IGFBP1), 60 °C (IGFBP4, IGFBP6), 63 °C (IGFBP5), and 52 °C glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for 20 s, extension at 72 °C for 30 s and fluorescence reading at 74 °C. All measurements for each sample were performed in duplicate. The efficiency of PCRs and relative quantities were determined from a six-point standard curve. Standard curves were constructed from a dilution series of pooled cDNAs (including six dilutions from 1/5 to 1/160), and PCR efficiency was calculated using the StepOne software v2.2. In standard curves, R^2 : 0.99, except in the case of GAPDH, where R^2 : 0.98. About 15 ng cDNA was used for all primers, except for IGFBP-1, where 90 ng was used for each real time PCR reaction, and was previously quantified by the Qubit method (Invitrogen). For amplification, 4 µl of cDNA was combined with a PerfeCta SYBR Green SuperMix, ROX (Quanta Biosciences, Inc., MD, USA) in a final volume of 20 µl. The primer sequences used are described in Table 1. The GAPDH gene sequence was included as the housekeeping gene.

The mRNA expression levels of genes were recorded as cycle threshold (Ct) values that corresponded to the number of cycles at which the fluorescence signal can be detected above a threshold value. The Ct was calculated manually using StepOne v2.2 (Applied Biosystems). Negative DNA template controls were included in all the assays, and yielded no consistent

173 amplification. Product purity was confirmed by dissociation curves, and random samples were
174 subjected to agarose gel electrophoresis. Fold change was determined using the $2^{-\Delta\Delta C_t}$ method
175 (Livak et al., 2001).

176 177 Nucleotide sequencing

178 The specificities of the PCR products were checked by direct sequencing to ensure
179 amplification of the correct sequences using the MacroGen Sequencing Service (MacroGen,
180 Korea). The resulting sequences were verified using the MegAlign Tool in the LASERGENE
181 software (DNASar, WI, USA).

182 183 In situ hybridization

184 All reagents and materials were RNase-free. Sections were prepared as described above,
185 but using RNase-free conditions. Single-stranded oligonucleotide probes of 45 bases, end-
186 labeled with biotin (Invitrogen) were used and, together with those detected previously
187 (Rodríguez et al., 2011), we assayed the main IGFBPs described in bovines. Probe sequences are
188 described in Table 2.

189 Probes were diluted to a final concentration of 200 ng/ml in a hybridization solution
190 (Sigma). The sections were dewaxed, hydrated and endogenous peroxidase activity was
191 inhibited with 1 % H_2O_2 in methanol. After washing with Tris-buffered saline (TBS) and sterile
192 water, sections were subjected to microwave pre-treatment in citrate buffer (pH 6.0). After
193 washing with TBS, the corresponding probe was added to each slide and the sections were
194 incubated in a humid chamber overnight at 45 °C (Llewellyn et al., 2007). Following incubation,
195 the slides were washed with TBS and a pre-warmed astringent solution (one wash of saline-

sodium citrate (SSC) 2X-50 % formamide-0.05 % Tween for 5 min, followed by two washes of SSC 2X for 10 min, one wash of SSC 1X for 10 min and one wash of SSC 0.1X for 15 min, all at 45 °C; finally one wash with SSC 0.1X for 5 min at room temperature). The slides were then rinsed with distilled water and TBS and incubated with extravidin-peroxidase (1:200, Sigma) for 30 min at 25°C. A positive reaction was visualized using 3,3'-diaminobenzidine (DAB, Liquid DAB-Plus Substrate Kit, Zymed, CA, USA) as the chromogen. Finally, the slides were washed in distilled water and then counterstained with Mayer's hematoxylin, dehydrated and mounted. To verify specificity, adjacent negative control sections were subjected to the same procedure, replacing the probe with hybridization solution or incubating with a sense probe. The same hybridization temperature, washing and detection conditions were used for the probes used.

Immunohistochemistry

Protein expression of IGFBP1, IGFBP4, IGFBP5 and IGFBP6 was detected using polyclonal antibodies (conditions in Table 3; Novozymes GroPep Ltd, Australia). The extravidin biotin immunoperoxidase method was carried out as described previously (Ortega et al., 2009; Salvetti et al., 2009). For antigen visualization, DAB was used as the chromogen. Negative control sections in which the primary antibody was replaced by a non-immune rabbit serum were included (Ortega et al., 2009). Some sections were incubated with DAB alone to exclude the possibility that endogenous peroxidase activity had been unsuccessfully blocked.

Western blotting

To test the specificity of the primary antibodies used, bovine tertiary follicles were homogenized in a radio-immunoprecipitation assay lysis buffer with a protease inhibitor cocktail

(Complete Mini Protease Inhibitor Cocktail Tablets, Roche, Mannheim, Germany), as described previously (Rodríguez et al., 2011). To this end, 40 µg of protein, along with pre-stained molecular weight markers (GE Healthcare Buckinghamshire, UK) were separated in SDS-PAGE containing 15 % (w/v) acrylamide-polyacrylamide, according to a procedure previously described (Ortega et al., 2009). After blotting on nitrocellulose membranes (Hybond ECL Nitrocellulose Membrane, GE Healthcare), the membranes were blocked with TBS containing 0.05 % v/v Tween20 (TBST, Sigma-Aldrich Corp.) and 5 % w/v non-fat milk, and then incubated overnight at 4°C with specific primary antibodies. Bound antibody was detected using anti-rabbit IgG peroxidase antibody (Amersham, Buckinghamshire, UK; 1:500) and ECL plus western blotting detection reagents (GE Healthcare).

Western ligand blot

Samples of follicular fluid were separated electrophoretically under non-reducing conditions and transferred onto a nitrocellulose membrane as described previously (Rey et al., 2010). Membranes were blocked with 5 % w/v bovine serum albumin (Sigma-Aldrich) in TBS and then incubated overnight at 4°C with 750 ng/ml of biotinylated IGF1 (Novozymes GroPep). Membranes were washed with TBST, and incubated with a 1:5000 dilution of extravidin-peroxidase (Sigma-Aldrich) in blocking solution. Before washes with TBST, signal was detected by chemiluminescence using the ECL-Plus system (GE Healthcare) on hyperfilm-ECL film (GE Healthcare). Individual binding proteins were identified on the basis of their molecular weights, as reported previously (Jones and Clemmons, 1995; Nicholas et al., 2002). The molecular weight reported for IGFBP3 corresponds to a double band of 40 and 44 kDa. IGFBP2 was detected at 34

kDa, IGFBP4 has been reported to have a glycosylated and a non-glycosylated form, with molecular weights of 29 and 24 kDa, respectively, and IGFBP5 was observed at 31 kDa.

Image analysis

Images were analyzed using the Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver Spring, MA, USA). Images were digitized using a color video camera (Motic 2,000, Motic China Group, China) mounted on top of a conventional light microscope (Olympus BH-2, Olympus Co., Japan) using an objective magnification of $\times 40$, as described and validated previously (Ortega et al., 2009, 2010; Rodríguez et al., 2011). To obtain quantitative data for IHC and ISH labeling of IGFBPs in the follicular wall, at least three sections were evaluated for each specimen and antibody or oligonucleotide. The average density (% of positive area) of the IGFBPs antibody or oligonucleotide reaction was calculated from at least 20 images of each area (granulosa and theca cells) in each section as a percentage of the total area evaluated through color segmentation analysis, which extracts objects by locating all objects of a specific color (brown stain) (Ortega et al., 2009).

For the western ligand blot, the exposed films were scanned at 1200 dpi (scanner HP Officejet J5, 780). The level of each IGFBP was analyzed by densitometry to obtain an integrated optical density (IOD) value, which was compared between follicular fluid samples from healthy and cystic ovaries (Rey et al., 2010).

Statistical analysis

A statistical software package (SPSS 11.0 for Windows, SPSS Inc., Chicago, Illinois, USA) was used to analyze the data. Tests of homogeneity of variance among groups were conducted

using Levene's test. The statistical significance of differences between groups of data was assessed by one-way ANOVA, followed by Duncan's multiple range tests as a multiple comparison test. A value of $P < 0.05$ was considered significant. The results are expressed as mean $\pm$ SD.

RESULTS

IGF1 and IGFBPs gene expression

The identity of the PCR products was confirmed by sequencing (100% homology with bovine sequences). No differences were detected in GAPDH gene expression between the different groups evaluated ($P > 0.05$).

Levels of mRNA of IGFBP1, IGFBP4, IGFBP5 and IGFBP6 in the complete follicular wall of control tertiary follicles of different sizes and cystic follicles were similar ($P > 0.05$) (Figure 1). Moreover, no differences were detected in the levels of IGF1 of tertiary follicles and cystic structures ($P > 0.05$) (Figure 2A). However, when mRNA expression was analyzed in different follicular cells by ISH, IGFBP5 was detected mainly in granulosa cells, where the expression determined in cystic structures was lower than that in growing follicles (Figure 3B and 4). Growing follicles from both control and cystic ovaries showed a decreasing expression from primary to tertiary follicles. IGFBP5 mRNA was detected in theca cells of cystic follicles, but a very low presence or complete absence was detected in theca cells of tertiary follicles.

IGFBP4 was detected in granulosa and theca cells, and no differences were observed in the structures analyzed ($P > 0.05$) (Figure 3). Similarly to that observed in mRNA expression of IGFBP5, in granulosa cells, IGFBP4 mRNA levels decreased as follicles grew, and the lowest levels were detected in cystic structures ($P < 0.05$).

IGF1 mRNA was detected only in granulosa cells, with similar levels between structures of the same group (control, spontaneous COD and experimentally induced COD). However, reduced levels were detected in growing follicles and cysts from ovaries with induced COD compared with control and spontaneous COD ($P < 0.05$).

Structures analyzed in follicles from ovaries with spontaneous COD consistently showed higher positive labeling than follicles from ovaries with experimentally induced COD, and the expression pattern was similar in each structure analyzed (Figure 2B, 3).

Protein expression of IGFBPs

Expressions of IGFBP1, IGFBP4, IGFBP5 and IGFBP6 were evaluated in follicles of different developmental stages. Insulin-like growth factor binding protein 4, IGFBP5 and IGFBP6 were detected both in granulosa and theca cells, with greater levels in granulosa cells (Figure 6), whereas IGFBP1 was not observed in either cell type.

Both IGFBP4 and IGFBP5 showed the lowest expression levels in granulosa cells of cystic structures, and the highest expression in primary follicles of control ovaries as well as in ovaries with COD ($P < 0.05$) (Figure 7). Theca cells of experimentally induced cystic follicles showed lower expression levels of IGFBP4 than control tertiary follicles ($P < 0.05$) and expression levels similar to those of spontaneous cystic follicles ($P > 0.05$). Expression levels of IGFBP5 were similar in the control and induced structures analyzed ($P > 0.05$), and different from those in spontaneous structures ($P < 0.05$).

In contrast, IGFBP6 expression in granulosa cells increased expression from primary to tertiary follicles, with larger levels in cystic follicles of experimentally induced COD ($P < 0.05$) (Figure 7). Once more, the levels were higher in the structures from spontaneous COD and

increased from primary to secondary follicles, and then remained constant through tertiary and cystic follicles. IGFBP6 was not detected in theca cells.

Follicular fluid content of IGFBPs

The content of IGFBPs in follicular fluid from different sized follicles was identified based on their molecular weight (Nicholas et al., 2002). The western ligand blot allowed for the identification of IGFBP2 (34 kDa), a doublet corresponding to IGFBP3 (40-44kDa), IGFBP4 in its glycosylated (29 kDa) and non-glycosylated forms (24 kDa), and IGFBP5 (31 kDa). Their identities were confirmed by western blot. The patterns for IGFBP2 and IGFBP3 have been shown in a previous work (Rodríguez et al., 2011), whereas those for IGFBP4 and IGFBP5 are shown in Figure 8.

IGFBP2 and IGFBP3 were expressed at higher levels in the follicular fluid of the structures analyzed than the other IGFBPs detected. However, while IGFBP2 levels remained practically constant in tertiary follicles and cysts ($P > 0.05$), IGFBP3 levels were higher in cysts than in small tertiary follicles ($P < 0.05$). In contrast, the glycosylated form of IGFBP4 and IGFBP5 showed higher levels in small tertiary follicles than in cysts and tertiary follicles greater in size ($P < 0.05$). No differences in the non-glycosylated form of IGFBP4 were detected in the follicles analyzed (Figure 8).

DISCUSSION

The study of the etiopathogenesis of COD in dairy cows has several limitations. The main difficulties are the fact that bovine COD is a multifactorial disease and there are few opportunities to follow clinical cases without treatment because farmers need their cows to

333 receive treatment as soon as possible (Ribadu, 2000; Probo et al., 2011). For this last reason,
334 many studies have been conducted with slaughterhouse material, which lacks the reproductive
335 history and information of cyst persistence. In the present study, we were able to follow the
336 reproductive behavior by ultrasonography and rectal palpation of the dairy cows identified as
337 having COD, and thus to promptly detect persistent follicles and development of cysts.
338 Moreover, since follicular cysts were recovered from experimental animals at known times after
339 cyst emergence, analysis of their structure and function was more accurate (Ortega et al., 2008).

340 In the present study, we determined that IGFBP4 and IGFBP5 mRNA levels in granulosa
341 cells decreased from primary to tertiary follicles in the control group, similarly to that observed
342 in sheep (Hastie et al., 2004; Hastie and Haresign, 2006). Interestingly, IGFBP4 mRNA was
343 detected mainly in granulosa cells, but expressed in lower levels in theca cells, contrarily to that
344 observed by other authors (Armstrong et al., 1998; Roberts and Etcherntkamp, 2003).
345 Moreover, the reduced levels of IGFBP4 and IGFBP5 detected in granulosa cells of cystic ovaries
346 were more evident in the experimental model than in spontaneous cysts, probably due to the
347 differences in the environmental conditions and the time of persistence of the cysts, which
348 could influence the gene expression of these IGFBPs. Consistently, IGFBP5 protein expression
349 showed a similar pattern. On the other hand, similar protein levels of IGFBP4 were observed in
350 the structures from ovaries from spontaneous and induced COD. Differences in mRNA levels
351 related to protein expression could be due to the hormonal milieu that participates in the
352 control of the IGFBP system and proteins associated such as IGFBP proteases (Spicer, 2004; Aad
353 et al., 2006; Sudo et al., 2007). Moreover, it is worth mentioning that levels detected by IHC
354 correspond to the glycosylated form of IGFBP4, as demonstrated by western blot.

In support of the control exerted by steroid hormones on IGFBP production, Spicer and Chamberlain (2000) observed that estradiol inhibited IGFBP4 production in bovine granulosa cells in vitro, but did not determine whether this inhibition was due to a change in the proteolysis of IGFBP4. IGF1, FSH, and/or estradiol induce IGFBP4 proteolysis in cultured human granulosa cells (Iwashita et al., 1998). In addition, the reduced levels of IGFBP4 and IGFBP5 determined in spontaneous and experimentally induced cystic follicles as compared with those in primary follicles from both control and COD ovaries are probably associated with the lower mRNA levels detected, although control by IGFBP proteases cannot be ruled out. It has been suggested that IGFBP4 production, via changes in intrafollicular mRNA expression, is correlated with LH regulation and IGFBP4 degradation (Mazerbourg et al., 2001; Hastie and Haresing, 2010). Moreover, both FSH and LH inhibit the expression of IGFBP5 mRNA in ovine follicles, determining a decrease in the levels of IGFBP5 mRNA (Besnard et al., 1996a; Hastie and Haresign, 2006a) and protein (Monget et al., 1993; Spicer and Chamberlain, 2002) in follicles as they increase in diameter (Hastie and Haresing, 2010).

Since there is limited information about the patterns of IGFBP1 and IGFBP6 mRNA and protein expression in the ovary of ruminants, little is known about the elements that regulate these binding proteins. In the present study, we found no changes in IGFBP1 and IGFBP6 mRNA levels in the follicular wall of the tertiary and cystic structures analyzed, and detected no protein expression in any of the follicles analyzed. Because lower levels of IGFBP1 and IGFBP6 mRNA were detected, not expression of those binding proteins mRNA were analyzed by situ hybridization. It has been reported that IGFBP1 concentrations in serum and follicular fluid are decreased in women with polycystic ovary syndrome (Thierry van Dessel et al. 1999). In bovines, IGFBP1 is detected in small quantities in the follicular fluid, probably because it is either poorly expressed

or not synthesized within the follicle compartment, entering the basal membrane as a result of changes (Nicholas et al. 2002). Probably, these changes could explain why we detected mRNA but not protein. This lack of detection of protein may also be due to the methodology used (IHC). This may also be true because we failed to detect both IGFBP1 and IGFBP6 in follicular fluid by ligand blot (Nicholas et al., 2002), but found IGFBP6 in granulosa cells.

Although IGFBPs were synthesized intrafollicularly (Armstrong et al., 1998; Voge et al., 2004; Rodríguez et al., 2011), we analyzed the levels of IGFBPs in follicular fluid, where IGF availability is effectively regulated. Changes in IGFBP concentrations in bovine follicular fluid have been documented during follicle growth and development in cattle (de la Sota et al., 1996; Stewart et al., 1996), sheep (Perks and Wathes, 1996), and pigs (Yuan et al., 1996). Typically, dominance is associated with decreased follicular fluid concentrations of the binding proteins with lower molecular weight, i.e. IGFBP4 and IGFBP5 (Austin et al., 2001), in agreement with our present results, which showed lower protein concentrations in tertiary follicles increasing in size. The changes in the IGFBP content of follicular fluid could be due to the regulation of IGFBP production, changes in the activity of specific IGFBP proteases, or changes in the selective uptake of IGFBPs from the circulation during follicle growth (Armstrong et al., 1998). In fact, we detected lower gene expression of IGFBP4 and IGFBP5, which was reflected in lower protein synthesis, while follicles were growing from primary to tertiary and cystic follicles. However, no changes were detected in mRNA or protein expression levels of control tertiary follicles when compared with spontaneous cystic follicles. In follicular fluid, a decreasing concentration of IGFBP5 was detected through tertiary follicles growing in size until cystic structures. Similar results were obtained with the 29-kDa glycosylated form of IGFBP4, although no changes were

detected with the 24-kDa non-glycosylated form. The lower levels detected could result from an increasing activity or concentration of IGFBP protease.

Previously, we detected similar protein expression of IGFBP2 and IGFBP3 in tertiary control and cystic follicles (Rodríguez et al., 2011). However, the concentration of IGFBP3 in follicular fluid increased as the control tertiary follicles increased in size, with the highest concentration found in cystic structures. Therefore, the differences detected in IGFBP3 content in follicular fluid support the hypothesis of an extrafollicular origin (Echternkamp et al., 1994; Santiago et al., 2005; Rey et al., 2010), probably responsible for the bioavailability of IGF1.

In previous studies, we detected similar serum IGF1 concentrations in controls and animals with spontaneous or induced cystic ovarian disease (Ortega et al., 2008). Part of the lack of a direct effect on ovarian activity depends on how much of the circulating IGF1 concentration is truly reflected in the ovaries. IGF1 concentration in follicular fluid has been found to be lower (Echternkamp et al., 1990; Spicer et al., 1991), equal to (Spicer et al., 1992), or greater (Spicer et al., 1992; Ortega et al., 2008) than peripheral IGF1 concentration, and although they are correlated, in some circumstances, such as short-term fasting, liver-derived IGF1 can be decreased significantly without affecting intrafollicular IGF1 concentration (Spicer et al., 1992; Velazquez et al., 2008). Furthermore, 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). In this pathological condition, while IGF1 concentrations are known to be lower during ovarian cyst formation (Beam and Butler 1997, 1998; Zulu et al. 2002), intrafollicular IGF1 can be even lower than serum concentrations (Ortega et al., 2008). Considering the alterations in the expression of IGFBP2 and IGFBP3 reported previously (Rodríguez et al., 2011) and the results of the present study, we suggest that the decreased IGF1 levels could result from

alterations in the diffusion rate between the blood and the follicular fluid. These alterations could be due to differences in the interaction with IGFBPs related to gene and protein expression and/or to induced changes attributable to a decreased IGF1 mRNA in ovaries with induced COD, causing an imbalance in the IGF system that could modify the circulating levels of the free fraction of IGF (Rechler and Clemmons, 1998; Hastie and Haresing, 2006; Thomas et al., 2007).

CONCLUSION

The results of the present study support that the IGF system has a clear influence in cows with COD. We suggest that IGF1 is influenced by the action of IGFBPs, mainly by IGFBP3 and by the decreased level of IGF1 mRNA during a COD condition.

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FIGURE LEGENDS

Figure 1: Expression of IGFBP1, IGFBP4, IGFBP5 and IGFBP6 mRNA in small (S), medium (M) and large (L) tertiary follicles and cysts (C). No differences were detected in the quantification of the PCR products of IGFBP1 (**A**), IGFBP4 (**B**), IGFBP5 (**C**) and IGFBP6 (**D**). Values represent the mean $\pm$ SD.

Figure 2: Expression of IGF1 mRNA levels in follicular structures. (**A**) Quantification of IGF1 mRNA in ovarian follicular wall of small (S), medium (M) and large (L) tertiary follicles and cysts (C) by real time PCR. No differences were detected in the PCR products of IGF1 mRNA. (**B**) Analysis of cell population by in situ hybridization showed decreased expression of IGF1 in experimentally induced cysts (black bars) related to control follicles (open bars) without differences in spontaneous cystic ovaries (hatched bars). Values represent the mean $\pm$ SD. Bars with different superscript letters denote significant differences ($P < 0.05$).

Figure 3: Expression of IGFBP4 and IGFBP5 mRNA levels by in situ hybridization. Quantification of IGFBP4 mRNA in granulosa and theca interna cells (**A**) and quantification of IGFBP5 mRNA in granulosa cells (**B**) in developing follicles of control ovaries (open bars), experimentally induced cystic ovaries (black bars) and spontaneous cystic ovaries (hatched bars). Values represent the mean $\pm$ SD. Bars with different superscript letters denote significant differences ($P < 0.05$). C: control, I: induced cystic ovaries, S: spontaneous cystic ovaries.

Figure 4: Representative images of IGFBP4 (**A-C**) and IGFBP5 (**D-F**) mRNA localization by in situ hybridization in bovine follicles. The immunoreactivity was intense in granulosa cells of tertiary control follicles (A, D) and weaker in cystic follicles (B, E). No staining was observed when the sense probe was pre-incubated with the antisense probe as negative control (C, F). In the right column, the immunopositive areas of A and D were segmented by digital image analysis, showing in black the differential cytoplasmic immunostaining pattern. Bars = 25 μ m

Figure 5: Representative images of IGF1 mRNA localization by in situ hybridization in bovine follicles. The immunoreactivity was intense in granulosa cells of secondary (**A**) and tertiary (**B**) control follicles and weaker in cystic follicles (**C**). No staining was observed when the sense probe was pre-incubated with the antisense probe as negative control (**D**). Bars = 25 μ m.

Figure 6: Representative images of IGFBP4 (**A-C**), IGFBP5 (**D-F**) and IGFBP6 (**G-I**) protein localization by immunohistochemistry in bovine follicles. The immunoreactivity was intense in granulosa cells and reduced in theca cells in the follicular structures analyzed. Images represent tertiary control follicles (A, D, G) and cystic follicles (B, E, H). No staining was observed when the primary antibody was replaced with normal rabbit serum (negative control C, F, I). Verification of antibody specificity by western blot analysis of an ovarian homogenate demonstrating the specificity of the antibody is shown on the left. In the right column, the immunopositive areas of A, D and G were segmented by digital

image analysis, showing in black the differential cytoplasmic immunostaining pattern.

Bars = 25 μ m

Figure 7: Immunohistochemical staining of IGFBP4 (**A**), IGFBP5 (**B**) and IGFBP6 (**C**), showing the percentage of immunopositive areas in granulosa and theca interna cells evaluated in primary, secondary, and tertiary follicles of control ovaries (open bars), experimentally induced cystic ovaries (black bars) and spontaneous cystic ovaries (hatched bars). Values represent the mean $\pm$ SD. Bars with different superscript letters denote significant differences ($P < 0.05$) in separate granulosa and theca cell compartments. The theca interna was not labeled for IGFBP6.

Figure 8: Detection of IGFBPs in follicular fluid by western ligand blot of healthy small (S), medium (M) and large (L) tertiary follicles compared with cystic follicles (C). **A:** Quantification of IGFBP subtypes as determined by molecular weight: IGFBP4 non-glycosylated form of 24 kDa (open bars), IGFBP4 glycosylated form of 29 kDa (squared bars), IGFBP5 (horizontally hatched bars) of 31 kDa, IGFBP2 (black bars) of 34 kDa and IGFBP3 (diagonally hatched bars). Values represent the mean $\pm$ SD. Bars with different superscript letters denote significant differences ($P < 0.05$) between different follicle types. **B:** Representative western ligand blot showing the IGFBPs that bind to IGF1.

Table 1: Forward and reverse primer sequences (5'→3')

Primer	Sequence (5'→3')	Length, bp	Reference
IGF1	For ¹ TCG CAT CTC TTC TAT CTG GCC CTG T Rev ² GCA GTA CAT CTC CAG CCT CCT CAG A	240	Pfaffl et al. (2002)
IGFBP1	For TCA AGA AGT GGA AGG AGC CCT Rev AAT CCA TTC TTG TTG CAG TTT	123	Pfaffl et al. (2002)
IGFBP4	For GCC CTG TGG GGT GTA CAC Rev TGC AGC TCA CTC TGG CAG	342	Plath- Gabler et al. (2001)
IGFBP5	For TGC GAG CTG GTC AAG GAG Rev TCC TCT GCC ATC TCG GAG	257	Plath- Gabler et al. (2001)
IGFBP6	For AGA AAG AGG ATT TGC CTT TGC Rev TCC GGT AGA AGC CCC TAT G	324	Plath- Gabler et al. (2001)
GAPDH	For CAC CCT CAA GAT TGT CAG CA Rev GGT CAT AAG TCC CTC CAC GA	103	Shibaya et al. (2007)

¹ Forward (For)² Reverse (Rev)

Table 2: Probe sequences (5'→3')

Probe	Sequence (5'→3')	Reference
IGF1	TCACATCCTCCTCGCATCTCTTCTATCTGGCCCTGTGCTTGCTCG	(Llewellyn et al., 2008)
IGFBP4	AAGACGGGAGTGAAGCTTCCGGGGGGCCTGGAGCCGAAGGG GGAG	901–945 of bovine IGFBP4 mRNA (Moser et al. 1992)
IGFBP5	CTACTCGCCCAAGATCTTCCGGCCCAAGCACACCCGCATCTCCG A	291–335 of bovine IGFBP5 gene (Moser et al. 1992)

Table 3: Antibodies, suppliers and dilutions used

Antibodies	Type	Supplier	Dilution
Primary antibodies			
IGFBP1	Polyclonal	Novozymes GroPep Ltd, Australia	--
IGFBP4	Polyclonal	Novozymes GroPep Ltd, Australia	1:100
IGFBP5	Polyclonal	Novozymes GroPep Ltd, Australia	1:75
IGFBP6	Polyclonal	Novozymes GroPep Ltd, Australia	1:200
Secondary antibody			
Anti-rabbit IgG	Polyclonal	Zymed, CA, USA	1:200

Figure 1

Figure 4

Figure 5

Figure 6

B