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SPONTANEOUSLY ARISING DISEASE

Involvement of Matrix Metalloproteinases and their Inhibitors in Bovine Cystic Ovarian Disease

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Summary

The most important regulators of tissue remodelling during ovarian follicular growth, development, ovulation and atresia are gonadotropins, steroid hormones, growth factors and different proteolytic enzymes. Matrix metalloproteinases (MMPs) such as collagenase or gelatinase (i.e. MMP-1, -8, -2 and -9) and associated tissue inhibitors of metalloproteinases (TIMP-1, -2, -3 and -4) control connective tissue remodelling during follicular rupture. In this study, we hypothesized that an imbalance in the MMP–TIMP system may be an intra-ovarian component that contributes to the pathogenesis of cystic ovarian disease (COD) in cows. Taking into account that the control of MMP activity by TIMPs could determine their effects in both physiological and pathological conditions, MMP and TIMP mRNA and protein expression was examined by real-time polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC), respectively, in ovaries from control cows and cows with COD. Expression of mRNA encoding MMP-2, TIMP-1 and TIMP-2 was lower in follicular cysts than in control pre-ovulatory follicles, while the results by IHC showed this imbalance only for TIMP-2 protein expression. Additional analysis by zymography to evaluate the gelatinase activity of MMP-2 and MMP-9 demonstrated higher MMP-2 activity in follicular fluid (FF) of cysts than in FF of pre-ovulatory follicles. On the other hand, MMP-9 activity was increased in follicular cysts and absent in the FF of pre-ovulatory follicles. These findings suggest that the altered mRNA expression and activities of the MMP–TIMP system may be related to the failure in ovulation and follicular development observed in COD.

Keywords: cow; matrix metalloproteinase; ovary; tissue inhibitors of metalloproteinase

Introduction

Ovulation is a synchronized and complex inflammatory process controlled by endocrine and biochemical events that cause follicle rupture and extrusion of the oocyte (Espey, 1994; Bukovsky and Caudle, 2008; Amweg *et al.*, 2013). During follicular development, ovulation and atresia, ovaries undergo continuous tissue remodelling. Specific components of the extracellular matrix (ECM) can be altered through cleavage by matrix metalloproteinases (MMPs), such as collagenase (i.e. MMP-1, -8 and -13) or gelatinase (i.e. MMP-2 and -9), whose activities are inhibited by tissue inhibitors of metalloproteinases (TIMPs) (Smith *et al.*, 2002). The extensive follicular remodelling is evident in the dissolution of the granulosa cell basement membrane that allows the oocyte release by fragmentation of the ECM of the follicular wall during ovulation (Richards *et al.*, 2002; Bilbao *et al.*, 2011). Moreover, the remodelling of the ECM through MMPs can affect multiple cellular processes such as proliferation, differentiation and apoptosis. Inhibition of MMPs by TIMPs can stabilize the

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components of the ECM and promote ECM deposition. The MMP:TIMP ratio may contribute to the control of many cellular processes associated with ovarian function. Additionally, TIMPs can act as growth factors through membrane-bound TIMP receptors (Smith *et al.*, 2002).

MMP-2 and -9 are identified by gelatin zymography as two distinct proteins of 72 and 92 kDa, respectively (McIntush and Smith, 1998; Curry *et al.*, 2001; Imai *et al.*, 2003). These enzymes are able to bind to and cleave gelatin and therefore degrade many constituents of basement membranes. Additionally, the activity of MMPs in the extracellular space is regulated by TIMPs.

TIMPs are strongly involved in cyclic ovarian processes (Curry and Osteen, 2003; Zhang *et al.*, 2003; Li *et al.*, 2004; Kliem *et al.*, 2007). TIMP-1, -2, -3 and -4, which differ in their regulation, enzyme specificity and mode of action, have been identified in different tissues of several species (Docherty *et al.*, 1985; Carmichael *et al.*, 1986; De Clerck *et al.*, 1989; Stetler-Stevenson *et al.*, 1989; Pavloff *et al.*, 1992; Greene *et al.*, 1996). TIMP-1 and -2 are the two most studied inhibitors. TIMP-1 can bind to the active forms of all known MMPs and the latent form of MMP-9 and may regulate steroidogenesis by stimulating progesterone production by rat granulosa cells. TIMP-2 is able to bind active MMPs and inhibit their protease activity (Boujrad *et al.*, 1995; Woessner, 2001; Zhang *et al.*, 2003). Although TIMP-2 has highest affinity for MMP-2, it may also be involved in proMMP2 activation, which indicates that this TIMP could have a dual function.

MMPs and TIMPs are differentially distributed in the ovary of rats, women and cows and the mRNA expression levels of MMPs and TIMPs change in association with gonadotropin-induced follicular development (Smith *et al.*, 1996; Duncan *et al.*, 1998; Simpson *et al.*, 2001). In this sense, the control of MMP activity by TIMPs could determine their effects in both physiological and pathological conditions (Curry and Osteen, 2003). However, in cattle, the role of MMPs in normal ovarian events, such as follicular growth, ovulation and/or atresia, is less known and there is no information about altered expression during disturbed repair/degradation mechanisms of the ECM in cystic ovarian disease (COD).

COD, which is considered one of the most important causes of reproductive failure in cattle, can result in significant economic losses to the dairy industry by delaying conception (Peter, 2004; Cattaneo *et al.*, 2014). This disease has been defined as the presence of one or more follicular structures in the ovary/ovaries, with a diameter of at least 20 mm, which persist for more than 10 days in the absence of luteal tissue, interrupting the normal reproductive cycle (Silvia *et al.*, 2002; Peter, 2004; Vanholder *et al.*, 2006). The pathogenesis of COD in dairy cattle is a complex process that involves dysfunction in folliculogenesis and ovulation, with an important intra-ovarian component (Ortega *et al.*, 2015). Based on this evidence, the aim of the present study was to determine the mRNA and protein expression patterns and activity of MMP-2 and MMP-9, and the expression of their inhibitors TIMP-1 and TIMP-2, in ovarian follicles from control cows and cows with COD. This study allowed us to test the hypothesis that an imbalance in the MMP−TIMP system may be a component of the pathogenesis of follicular persistence and ovulatory failure in this reproductive disorder.

Materials and Methods

Ethical Aspects

All procedures were carried out according to the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (2010) and with the approval of the Institutional Ethics and Security Committee (Protocol N° 84/11, Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, Santa Fe, Argentina).

Animals

All cows were from local dairy farms. Twenty multiparous (66.2 ± 25.4 months old; 2.8 ± 1.3 lactations) Holstein cows, without reproductive disorders, were assigned to the control group ($n = 20$). Oestrous cycles were synchronized using the Ovsynch™ protocol, as previously described (Ortega *et al.*, 2008; Amweg *et al.*, 2013). Normal folliculogenesis was monitored daily by ultrasonography, using a real-time B-mode scanner equipped with a 5 MHz linear-array transrectal transducer (Honda HS101V, Toyohashi, Japan), through a complete oestrous cycle, to obtain normal growing follicles approximately on day 18 (Díaz *et al.*, 2015). The ovaries from 10 control cows were removed by transvaginal ovariectomy when the dominant follicle reached a diameter >10 mm, and the ovaries were macroscopically examined (Marelli *et al.*, 2014). The remaining 10 cows were subjected to follicular aspiration of dominant ovulatory follicles, using a digital ultrasound system Chison 8300vet equipped with a microconvex 5.0 MHz transducer (Chison Medical Imaging Co., Wuxi, China) mounted on a transvaginal probe for follicular fluid (FF) aspiration (Watanabe Applied Technology Limited., Sao Paulo, Brazil) (Marelli *et al.*, 2014).

Twenty lactating Holstein cows with spontaneously arising cystic follicles (64.8 ± 24.9 months old; 3.3 ± 1.5 lactations) were selected to obtain ovaries by transvaginal ovariectomy or FF by follicular aspiration. The cows had been monitored and the presence of follicular cysts had been confirmed by transrectal ultrasonography. Spontaneously arising follicular cysts were identified as one follicular structure of 20 mm in diameter persisting for at least 10 days in the absence of a corpus luteum (Bartolomé *et al*., 2005; Vanholder *et al*., 2006). Ten cows with COD were subjected to ovariectomy (Marelli *et al*., 2014). FF from the remaining 10 cows with COD was aspirated from cystic follicles and maintained under refrigeration until processed.

After excision, the ovaries and FF from both groups (control and COD cows) was refrigerated and immediately transported to the laboratory for processing, as described below.

Tissue Sampling and Processing

The ovaries were fixed in 4% neutral buffered formaldehyde at room temperature for 8−12 h. The fixed tissues were washed in phosphate buffered saline (PBS), dehydrated and embedded in paraffin wax. Sections (4 μm) were mounted on slides previously treated with 3-aminopropyltriethoxysilane (Sigma−Aldrich, St. Louis, Missouri, USA) and stained with haematoxylin and eosin (HE).

Additionally, small samples of ovarian tissue were immediately frozen at −80°C until used in western blotting to determine the specificity of the antibodies used in immunohistochemistry (IHC) (Velázquez *et al*., 2010).

FF was centrifuged to separate cells. Both cells and FF were stored at −80°C until RNA extraction or hormonal determinations/gelatin zymography, respectively.

Blood samples were obtained from all cows to test the hormone concentrations. The health status of the follicles was confirmed by the hormonal concentrations measured in the FF. These data have been published previously in parallel studies (Ortega *et al*., 2008; Amweg *et al*., 2013). The absence of additional diseases and abnormalities in the reproductive system was confirmed clinically.

RNA Isolation and Reverse Transcription

Total RNA was extracted from follicular cell pellet using TRIzol reagent (Life Technologies, Carlsbad, California, USA) according to the manufacturer's instructions. The RNA pellets were dissolved in nuclease-free water and yields of RNA were measured by spectrophotometry using SPECTROstar Nano (Life Technologies). Before reverse transcription, RNA samples were treated with DNAse I (Life Technologies) according to the manufacturer's instructions to eliminate possible genomic contamination (Baravalle *et al*., 2015). Total RNA was reverse transcribed to single-stranded cDNA, using a master mix containing Moloney murine leukemia virus (MMLV), buffer, dithiothreitol, ribonuclease inhibitor (RNA-OUT™), MMLV reverse transcriptase, deoxyribonucleotide triphosphate (dNTP) and random primers (Life Technologies). The reaction was carried out as described previously (Rodríguez *et al*., 2011). The concentration of cDNA in each sample was measured by spectrophotometry. All cDNA samples were stored at −20°C for subsequent quantitative real-time polymerase chain reaction (RT-PCR) analyses.

Quantitative Real-time Polymerase Chain Reaction

Measurements of mRNA levels were based on SYBR® I technology and carried out by relative quantitative RT-PCR using a StepOne™ real-time PCR (Life Technologies) (Rodríguez *et al*., 2011). Primers were designed using Primer-Quest™ software (http://www.idtdna.com/Primerquest/Home/Index). The conditions of the optimized RT-PCR protocol for each primer pair are detailed in Table 1.

RT-PCR was performed in duplicate 20 μl reaction volumes consisting of 4 μl of cDNA (500 ng/ml), 4 μl of 5× Phire reaction buffer, 0.5 μl of each 10 μM forward/reverse primers, 0.2 mM dNTPs, 1 μl SYBR Green I (Life Technologies), 0.05 μl Phire Taq polymerase (Thermo Fisher Scientific Company, Vantaa, Finland) and appropriate volumes of sterilized DEPC-treated water.

Cytochrome P450 aromatase (*CYP19a1*) and cytochrome P450 17-hydroxylase/17, 20-lyase (*CYP17a1*) gene sequences were amplified to confirm bovine granulosa and theca cell mRNA in all samples, following protocols described previously (Velázquez *et al*., 2011) (Table 1). Only samples that were positive for *CYP19a1* and *CYP17a1* mRNA were considered for the study.

The amounts of cDNA used in the assays were determined from independent experiments where each standard curve for MMP-2, MMP-9, TIMP-1 or TIMP-2 was performed including serial dilutions of pooled cDNAs as template. The β-actin standard curve (a six point serial dilution) was performed and expression of β-actin mRNA was determined in each sample. Each experimental sample and each point on the standard curves were run in duplicate. Efficiency of PCRs and relative amounts were

Table 1
Primer pairs used for real-time polymerase chain reaction

Name	*Sequence (5′-3′)*	*Gene accession number*	*Amplicon size*	*Annealing temperature (°C)*	*Reference*
TIMP1					
Forward	TCGTGGGGACCGCAGAAGT	NM_174471.3	134 bp	60	This study
Reverse	CTCCATGGCAGGGGTGTAGAT				
TIMP2					
Forward	CCCATCAAGCGGATTCAGTAT	NM_174472.3	136 bp	62	This study
Reverse	ACTCCTTCTTTCCTCCAATGTC				
MMP2					
Forward	TCTTCGCCGGAGACAAATTCTGGA	NM_174745.2	133 bp	60	This study
Reverse	ATCCAGGTTATCAGGGATGGCGTT				
MMP9					
Forward	AGGGTAAGGTGCTGCTGTTC	NM_174744.2	137 bp	62	This study
Reverse	CTGAAAGATGTCGTGCGTGC				
CYP17A1					
Forward	GGAGGCGACCATCAGAGAAGTGC	NM_174304	319 bp	60.8	Lagaly *et al.* (2008)
Reverse	CAGCCGGGACATGAAGAGGAAGAG				
CYP19A1					
Forward	TAAAACAAAGCGCCAATCTCTACG	BTCYP19	341 bp	55.4	Lagaly *et al.* (2008)
Reverse	GGAACCTGCAGTGGGAAATGA				
β-actin					
Forward	CGGAACCGCTCATTGCC	BT030480	290 bp	60	Riollet *et al.* (2001)
Reverse	ACCCACACTGTGCCCATCTA				

TIMP, tissue inhibitor of metalloproteinases; MMP, matrix metalloproteases; CYP17A1, cytochrome P450 17-hydroxylase/17, 20-lyase; CYP19A1, cytochrome P450 aromatase; bp, base pair.

determined from these standard curves. To corroborate PCR conditions, standard curves prepared with serial dilutions of pooled cDNAs from complete follicular wall were obtained for MMP-2, MMP-9, TIMP-1, TIMP-2 and β-actin as described above. These curves allowed us to obtain valuable information about the dynamic range of the template that yielded similar amplification efficiency. The PCR reaction efficiencies were 105% for MMP-2, 105% for MMP-9, 93.5% for TIMP-1, 94.6% for TIMP-2 and 93.44% for β-actin. The linearity in all curves of amplification was confirmed by fit with $R > 0.99$.

The relative expression of mRNA encoding MMP-2, MMP-9, TIMP-1 and TIMP-2 was calculated using a relative quantification method, which determines the expression of the target gene in comparison with a constant amount of β-actin mRNA as reference gene (Robinson *et al.*, 2007). The β-actin gene was previously validated as a better housekeeping gene for RNA analysis in bovine preovulatory and cystic follicles (Baravalle *et al.*, 2015). Negative DNA template controls were included in all assays. Product purity was demonstrated by dissociation curves, and random samples were subjected to agarose gel electrophoresis to verify the product sizes.

Nucleotide Sequencing

The specificities of the PCR products were confirmed by direct sequencing using the Macrogen Sequencing Service (Macrogen, Seoul, Korea). The resulting sequences were then tested using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) to confirm gene specificity (range 97–99% homology with bovine reference sequences).

Immunohistochemistry

Paraffin wax-embedded ovarian sections from cows with spontaneously arising follicular cysts and oestrous-synchronized control cows were subjected to immunohistochemical analyses to localize *in situ* the MMP-2, MMP-9, TIMP-1 and TIMP-2 proteins.

IHC was carried out using the streptavidin–biotin immunoperoxidase method (Velázquez *et al.*, 2010). Briefly, serial sections (4 μm) were mounted onto 3-aminopropyltriethoxysilane-coated slides. After dewaxing and rehydration, antigen was retrieved by incubating the sections in 0.01 M citrate buffer (pH 6.0) in a microwave oven at 800 W, except those for MMP-9 detection, which were pretreated by boiling in 0.001 M EDTA buffer (pH 8.0) with 0.05% Tween 20. The slides were treated for 20 min with 3% (v/v) H_2O_2 in methanol to eliminate endogenous peroxidase activity and non-specific binding was blocked with 10% (v/v) normal goat or normal donkey serum. Primary antibodies were incubated overnight at 4°C and detection was performed with the CytoScan™ HRP-Detection System (Cell Marque, Rocklin, California, USA) for 30 min at 25°C. 3′3-Diaminobenzidine (DAB; Biogenex, Fremont, California, USA) was used as chromogen. Conditions of the IHC and

suppliers of the primary antibodies used are reported in Table 2.

Parallel negative control sections with omission of the primary antibody and replacement of the primary antibody with non-immune rabbit, mouse or goat 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. Finally, the slides were washed in distilled water, counterstained with haematoxylin, dehydrated and mounted (Velázquez *et al.*, 2010).

Western Blotting

The specificity of the antibodies was tested with homogenates of ovarian follicular samples. Lysis buffer consisting of 1% v/v IGEPAL CA630 (octylphenyl-polyethylene glycol), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1 mM EDTA, 50 mM sodium fluoride (all from Sigma), 0.1 M PBS and a protease inhibitor cocktail (Complete Mini Protease Inhibitor Cocktail Tablets, Roche, Mannheim, Germany) was used to homogenize samples. Follicle homogenates were centrifuged at 14,000 g for 20 min and the supernatant was stored frozen at −80°C. Proteins (40 μg) were separated by SDS polyacrylamide gel electrophoresis (PAGE) (10% or 15% resolving gel), where prestained SDS-PAGE protein standards (GE Healthcare, Buckinghamshire, UK) were also loaded into an adjacent lane (Ortega *et al.*, 2009; Salvetti *et al.*, 2010; Velázquez *et al.*, 2011, 2013). Polyacrylamide gels were transferred onto nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). Non-specific binding sites were blocked with 5% non-fat milk in Tris-buffered saline containing 0.05% Tween-20 (Sigma) for 5 h and then incubated overnight at 4°C with the specific primary antibodies (Table 1). Following washing, membranes were treated for 1 h with the corresponding secondary peroxidase-conjugated antibody (Table 2). The immunopositive bands were detected by chemiluminescence, using the ECL-plus system (GE Healthcare) on hyperfilm-ECL film (GE Healthcare) (Velázquez *et al.*, 2010). MMP-2 was detected as a specific band at 63 kDa, MMP-9 as a specific band at 92 kDa, TIMP-1 as a specific band at 23 kDa and TIMP-2 as a single band at 24 kDa (Fig. 2).

Image Analysis

For each protein (MMP-2, MMP-9, TIMP-1 and TIMP-2), at least three sections for each specimen and antibody were used. Control antral follicles (as a reference structure) and follicular cysts were evaluated.

Images were captured with a digital camera (Nikon DS-Fi2, Tokyo, Japan) mounted on a light microscope (Nikon Eclipse Ci-L *Ni*). Images were analyzed using the Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver Spring, Massachusetts, USA) as previously detailed (Ortega *et al.*, 2010; Velázquez *et al.*, 2010). Briefly, the average density (percentage of positive area) of the antibody 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 colour segmentation analysis, which extracts objects by locating all objects of a specific colour (e.g. brown stain). All of the images analyzed covered granulosa cells from antrum to theca (Velázquez *et al.*, 2010).

Table 2
Antibodies, suppliers and dilutions used for immunohistochemistry and western blotting

Antibody	*Clone*	*Source*	*Dilution IHC*	*Dilution WB*
Primary				
TIMP-1	Rabbit polyclonal sc-6832R	Santa Cruz Biotechnology Inc., Santa Cruz, California, USA	1 in 100	1 in 250
TIMP-2	Mouse monoclonal ab1828	Abcam, Cambridge, Massachusetts, USA	1 in 100	1 in 400
MMP-2	Rabbit polyclonal sc-8835	Santa Cruz	1 in 400	1 in 3,000
MMP-9	Goat polyclonal sc-6840	Santa Cruz	1 in 50	1 in 250
Secondary				
Polyclonal HRP-anti-rabbit IgG	HRP-anti-rabbit IgG sc-2004	Santa Cruz	–	1 in 7,500
Monoclonal HRP-anti-mouse IgG	HRP-anti-mouse IgG sc-2005	Santa Cruz	–	1 in 6,000
Goat anti-rabbit IgG	Goat anti-rabbit sc-2040	Santa Cruz	1 in 100	–
Donkey anti-goat IgG-B	Donkey anti-goat Sc-2042	Santa Cruz	1 in 100	1 in 80,000

IHC, immunohistochemistry; WB, western blot; TIMP, tissue inhibitor of metalloproteinase; MMP, matrix metalloproteinase.

Gelatin Zymography

MMP activity in FF was determined by gelatin zymography in polyacrylamide gels. Briefly, all samples were analyzed for protein concentration by the Bradford method (Bradford, 1976). Aliquots of 100 mg of each sample were mixed briefly with sample buffer and loaded onto a 10% SDS-PAGE gel with 0.2 % porcine skin gelatin (Sigma). After electrophoresis, gels were washed and incubated with a solution containing 50 mM Tris−HCl, 10 mM $CaCl_2$ and 200 mM NaCl pH 7.5 at 37°C for 24 h. Gels were stained with 0.2% (w/v) Coomassie Brilliant Blue R-250 for 2 h and then bleached with decolourizing solution (25% v/v isopropanol plus 10% v/v acetic acid). Inhibition controls were performed by incubating with 5 mmol/l EDTA. The densitometry of the bands generated by the gelatinolytic activity was studied using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). Zymographic activity was expressed as a percentage of the control group. Data for different gels were standardized with the help of control samples (De Simone *et al.*, 2015). The migration of bovine MMP-2 and -9 was similar to that of the commercial marker of MMP-2 and -9 on the zymogram.

Statistical Analysis

Relative expression of mRNA encoding MMPs and TIMPs was calculated by REST 2009 V2.0.13 software (Pfaffl *et al.*, 2002), which uses average values of efficiency and cycle threshold (Ct) of target and reference genes. This software compares control and treatment Ct values to obtain the concentration of expression and performs a pairwise fixed reallocation randomization test (bootstrap = 2,000 permutation) to obtain P values.

The statistical software package SPSS 15.0 for Windows (SPSS Inc., Chicago, Illinois, USA) was used to analyse the results of IHC and gelatin zymography. Normal distribution was evaluated using the Kolmogorov−Smirnov test. The percentages of immunopositive area in the control group and animals with COD were compared by Student t-tests to determine differences between follicular categories. Differences were considered as significant when $P < 0.05$.

Results

Expression of mRNA Encoding MMPs and TIMPs

Expression of mRNA encoding MMP-2 was detected in the follicular samples and was higher in control pre-ovulatory follicles than in follicular cysts ($P < 0.05$) (Fig. 1). All of the follicular samples had weak

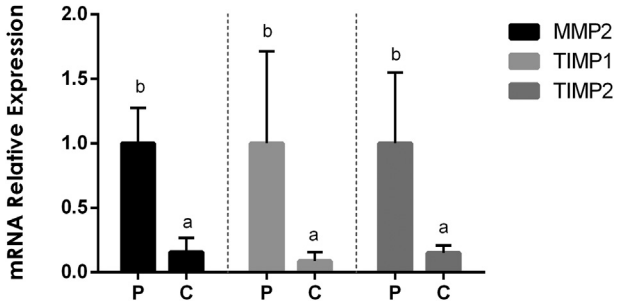

Fig. 1. Relative expression of mRNA encoding MMP-2, TIMP-1 and TIMP-2 in pre-ovulatory follicles (P) from control cows and cysts (C) from cows with COD. Values represent the mean ± SEM. Bars with different letters show significant differences ($P < 0.05$).

MMP-9 mRNA expression, and, since 75% of the samples expressed this gene outside the linear dynamic range, its relative quantification could not be performed.

TIMP-1 mRNA expression was detected in all follicular samples and was higher in control samples than in follicular cysts ($P < 0.05$) (Fig. 1). TIMP-2 mRNA expression was higher in pre-ovulatory follicles than in cystic follicles ($P < 0.05$) (Fig. 1).

MMP and TIMP Protein Expression

Protein expression was detected and localized in both control pre-ovulatory follicles and cysts by IHC. Representative images are shown in Fig. 2. Negative controls showed no immunolabelling for any of the follicular categories.

MMP-2 expression was detected in both the cytoplasm and some nuclei of granulosa and theca follicular cells. This labelling showed no significant differences between cystic and pre-ovulatory follicles ($P > 0.05$) (Figs. 3A, B).

MMP-9 expression was observed in the cytoplasm of granulosa and theca follicular cells, with similar expression levels between control and cystic follicles ($P > 0.05$) (Figs. 3A, B).

TIMP-1 expression was found in the cytoplasm of both granulosa and theca cells from cysts and pre-ovulatory follicles. No significant differences in the percentage of immunopositive area between groups were detected (Figs. 3A, B).

TIMP-2 expression was higher in the cytoplasm of pre-ovulatory than in cystic follicles, both in granulosa and theca cells ($P < 0.05$) (Figs. 3A, B).

Activity of MMP-2 and MMP-9 in Follicular Fluid

MMP-2 gelatinolytic activity was detected in all FF samples from the pre-ovulatory and cystic follicles (Fig. 4A). Zymographic analysis revealed higher

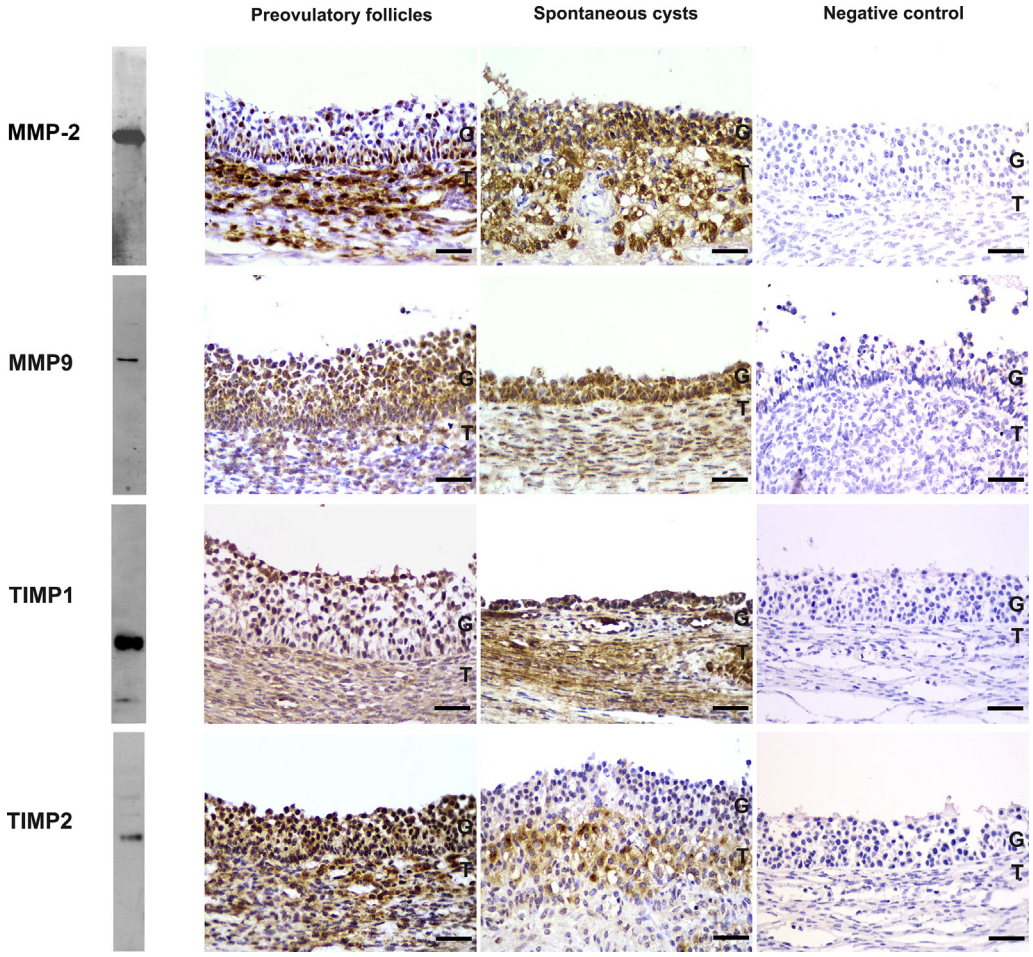

Fig. 2. Representative images of MMP-2, MMP-9, TIMP-1 and TIMP-2 immunolabelling in granulosa (G) and theca cells (T) of pre-ovulatory follicles from control cows and spontaneously arising cysts from cows with COD. Negative controls demonstrating the specificity of the antibody are also shown. Verification of antibody specificity by western blot analyses of ovarian homogenate is shown in the left column. Bars, 25 μm.

activity for MMP-2 in FF from cysts than in that from control pre-ovulatory follicles ($P < 0.05$) (Fig. 4B). In contrast, enzymatic activity for MMP-9 was observed only in cystic follicles.

Discussion

The results of the present study demonstrated that the MMP—TIMP system is altered in follicular cysts from cows with COD. In agreement with several studies that have shown that proteases are present in cell nuclei, in the present study, MMP-2 immunoexpression was observed in this compartment (Curry *et al.*, 2001; Sinha *et al.*, 2014). Although its functions, regulation and substrates remain unknown, recent reports have shown that the catalytically active gelatinase MMP-2 is expressed in the nuclei of endothelial cells and neurons (Sinha *et al.*, 2014).

Recently, Mutlag *et al.* (2014) found specific positive immunolabelling for MMP-2 in ovaries from fertile cows, but not in those from infertile cows obtained from slaughterhouses. In agreement with these results, we determined differences in the MMP2 mRNA expression between control and cystic follicles. However, IHC showed similar MMP-2 immunolabelling between control pre-ovulatory follicles and cysts from cows with spontaneously arising COD, which could be due to the post-translational processing of these proteins.

Although MMP gelatinase activity has been detected in cultured bovine theca and granulosa cells (Smith *et al.*, 2005), the factors that control the expression of these proteins are unknown. Previous reports have revealed that MMP-2 activity increases in the FF from pre-ovulatory follicles of cows when these structures reach maximum size (Imai *et al.*, 2003). In the present study, we detected gelatinase activity of MMP-2 in FF from control pre-ovulatory follicles, but the increased MMP-2 activity in cysts provides evidence about its functionality in follicles from cows with

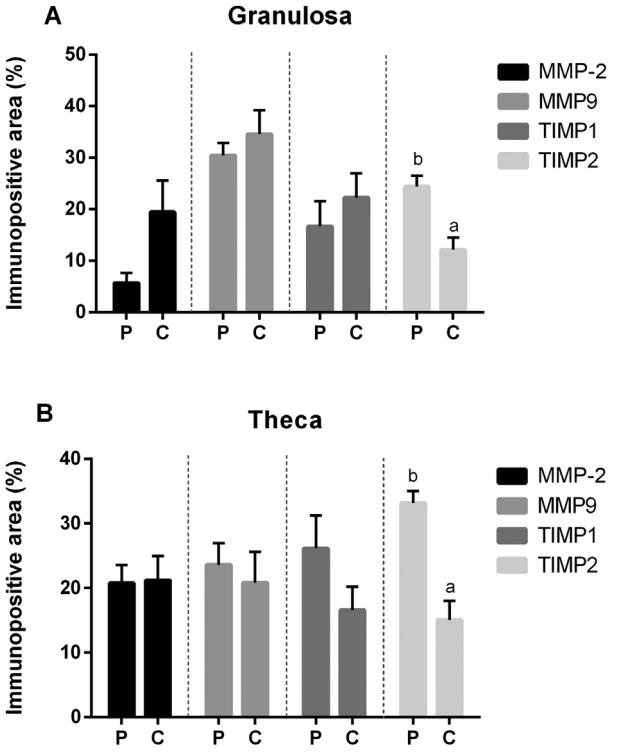

Fig. 3. Relative protein expression (measured as percentage of immunopositive area) of MMP-2, MMP-9, TIMP-1 and TIMP-2 in (A) granulosa and (B) theca cell layers of pre-ovulatory follicles (P) of control cows and spontaneously arising cysts (C) from cows with COD. Values represent the mean ± SEM. Bars with different letters show significant differences ($P < 0.05$).

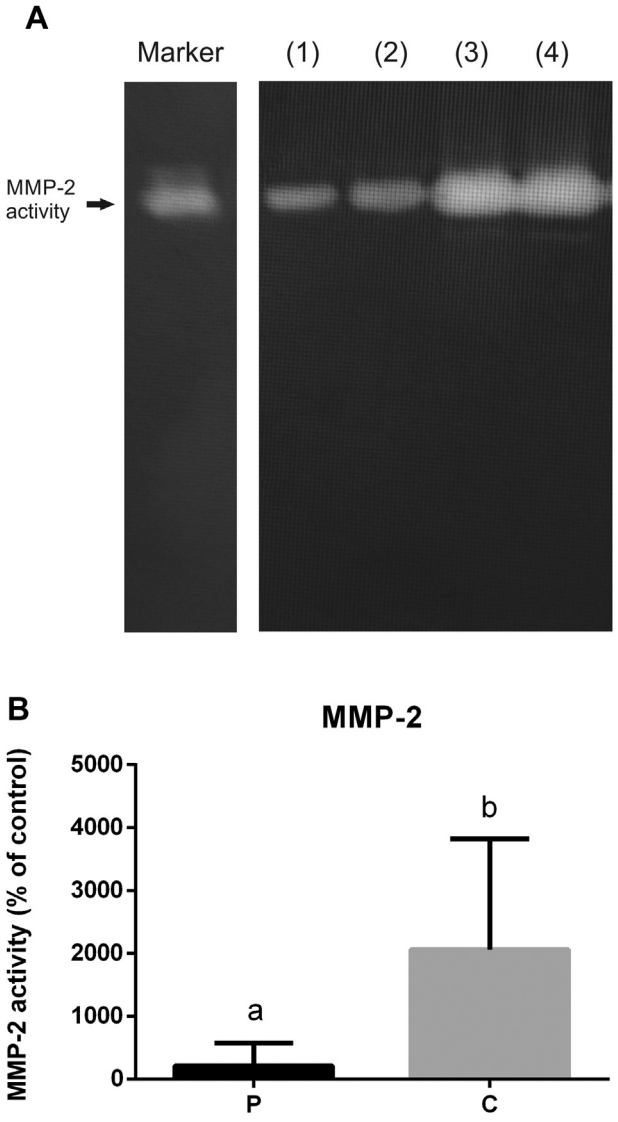

Fig. 4. (A) Representative image of gelatin zymography for detection of MMP-2 activity in pre-ovulatory follicles (lanes 1 and 2) and cystic follicles (lanes 3 and 4). (B) MMP-2 activities in follicular fluid from pre-ovulatory (P) and cystic (C) follicles (measured as percentage of activity in relation to control). Values represent the mean ± SEM. Bars with different letters show significant differences ($P < 0.05$).

COD. Many authors have suggested a significant role for MMPs in the growth and development of bovine follicles. In the FF, pro-MMP-2 and active MMP-2 could serve as markers of follicular health (Curry and Osteen, 2003; Imai *et al.*, 2003). Conversely, our results suggest increased activity in cystic follicles and suggest that MMP-2 activity in FF might not be an appropriate marker of healthy follicles.

MMP-9 protein and mRNA expression levels have been described in mice (Inderdeo *et al.*, 1996), rats (Cooke *et al.*, 1999), goats (García *et al.*, 1997) and ewes (Murdoch *et al.*, 1986). While pro-MMP-2 has been repeatedly detected in all sizes of ovarian follicles, MMP-9 has occasionally been found in FF of cows (Imai *et al.*, 2003). In agreement with previous studies (Imai *et al.*, 2003), here we observed no changes in protein levels between control pre-ovulatory and cystic follicles. The remarkable gelatinolytic activity of MMP-9 found in cystic follicles suggests a role in cyst formation, as previously reported by other authors (Baka *et al.*, 2010).

Studies of bovine follicles have demonstrated that MMP-2 and MMP-9 enzyme activities and mRNA levels are significantly higher in atretic than in healthy follicles (Yahia Khandoker *et al.*, 2001) and lower in healthy granulosa cells under the influence of follicle-stimulating hormone or insulin-like growth factor-1 *in vitro* (Portela *et al.*, 2009). These findings, together with the results presented here, highlight a possible association between unhealthy conditions of follicles and increased MMP gelatinase activity.

Recent findings have demonstrated increasing expression of TIMP-1 mRNA in theca and granulosa

cells, following the variation of the follicle size. Immunohistochemical analyses have revealed highest expression of TIMP-1 in the theca and granulosa cells of large antral follicles and corpora lutea from goats (Peng *et al.*, 2015). Our results partially support these findings since TIMP-1 mRNA expression was higher in pre-ovulatory follicles than in follicular cysts ($P < 0.05$). However, no evidence of those changes was found for TIMP-1 protein expression. Regarding the peri-ovulatory period, Zhang *et al.* (2003) have shown that TIMP-1 mRNA expression is increased during the early luteal phase and decreased ($P < 0.05$) from the mid to late stage in cows. Recently, Schmidt *et al.* (2014) found underexpression of TIMP-1 transcripts in ovarian stroma from women with polycystic ovary syndrome, which is in agreement with the results of the present work and might be related to a possible downregulation of MMP-9 expression in cysts and the lack of detection of mRNA in this study.

This is the first report about the expression pattern and localization of TIMP-2 in ovarian follicles from cows with spontaneously arising COD. Our results demonstrated lower TIMP-2 mRNA and protein expression in the ovaries of cows with COD than in those of the control group, both in granulosa and theca cells. TIMP-2 has been characterized as an important remodelling factor during the peri-ovulatory period and subsequent luteal phase (Smith *et al.*, 1996). It has been demonstrated that TIMP-2 activity increases in response to the gonadotropin surge, supporting the hypothesis that its expression may help regulate the restructuring of the ovarian ECM, follicle rupture and the transition to corpus luteum and its function (Goldman and Shalev, 2004). In the present study, TIMP levels were closely related to the changes in MMPs, as previously reported (Lind *et al.*, 2006; Baka *et al.*, 2010). High TIMP levels are needed to inhibit MMP activity (Lind *et al.*, 2006).

Considering the results obtained, we may conclude that there is a similar protein expression of MMP-2 in cysts and control pre-ovulatory follicles, and that MMP-2 is responsible, along with other enzymes, for the degradation of the follicular wall to promote ovulation. However, this is regulated by an increase in MMP-2 and MMP-9 enzyme activity in cysts, in relation to the pre-ovulatory follicles. In turn, this is accompanied by a decrease in the expression of the inhibitor, TIMP-2, which balances the system, reducing the possibilities of degradation of the follicular wall and hence favouring anovulation and possibly follicular persistence. Further studies of TIMP activities are necessary to confirm this hypothesis.

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Conflict of Interest Statement

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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