J. Comp. Path. 2018, Vol. 158, 22–31

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SPONTANEOUSLY ARISING DISEASE

Altered Expression of Anti-Müllerian Hormone during the Early Stage of Bovine Persistent Ovarian Follicles

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Summary

Anti-Müllerian hormone (AMH) is a homodimeric glycoprotein expressed exclusively in the gonads. This hormone is an important regulator of the early growth of follicles through inhibitory effects on the recruitment of primordial follicles into the pool of growing follicles and on granulosa cell proliferation. Cystic ovarian disease (COD) is an important disorder affecting the fertility of dairy cattle. In the present study, we evaluated the expression of AMH in granulosa cells and AMH secretion into follicular fluid in pre-ovulatory follicles from control cows, animals with spontaneously arising COD and during the development of the disease, at 5, 10 and 15 days of follicular persistence. To this end, after an oestrous synchronization protocol, low doses of progesterone was administered for 5, 10 and 15 days after the expected day of ovulation (day 0 of follicular persistence) in treated cows (groups P5, P10 and P15, respectively), using an intravaginal progesterone-releasing device. Results showed a decrease in the expression of AMH in granulosa cells throughout folliculogenesis ($P < 0.05$) and in the spontaneously arising follicular cysts and persistent follicles related to the control group ($P < 0.05$). There was also a higher concentration of AMH in the follicular fluid of persistent follicles at 10 and 15 days of follicular persistence ($P < 0.05$). Together, these results may indicate an alteration in AMH expression and secretion, which occurs early in folliculogenesis and incipiently during the development of COD, and which could contribute to the recurrence of this disease in cattle.

Keywords: anti-Müllerian hormone; cow; cystic ovarian disease; follicular persistence

Introduction

Cystic ovarian disease (COD) is an important disorder affecting the fertility of dairy cattle. It is characterized by follicles that fail to ovulate, persist for more than 6 days in the ovary and achieve a diameter of at least 20 mm (larger than the diameter of an ovulatory follicle), in the absence of a corpus luteum, with lack of uterine tonicity and interruption of the normal oestrous cycles (Silvia *et al.*, 2002; Bartolomé *et al.*, 2005; Ortega *et al.*, 2015). The incidence of COD ranges from 5% to 30%, depending on the herd where the problem appears. Although the main component of the aetiopathogenesis of COD is failure in the hypothalamic–pituitary–ovarian axis, there are important intraovarian components involved in the follicular persistence associated with the lack of ovulation (Matiller *et al.*, 2014; Ortega *et al.*, 2015, 2016).

At present, numerous ovarian extracellular cytokines have been identified and implicated in the autocrine/paracrine bidirectional communication between the oocyte and its surrounding somatic cells, as well as in the regulation of follicle survival, development and

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0021-9975/$ - see front matter
https://doi.org/10.1016/j.jcpa.2017.10.175

apoptosis. Members of the transforming growth factor-β superfamily are fundamental factors implicated in this dialogue (Knight and Glister, 2006; Field *et al*., 2014). One of the important members of this family is anti-Müllerian hormone (AMH), a homodimeric glycoprotein of 140 KDa expressed exclusively in the gonads. This hormone is an important factor in male sex differentiation, produced by Sertoli cells of the testis from fetal life until puberty, which promotes regression of Müllerian ducts during the differentiation of the male reproductive tract (Josso *et al*., 2001; Knight and Glister, 2006). In females, AMH is expressed in the granulosa cells of non-atretic pre-antral and small antral follicles and its expression is less evident in large antral and atretic follicles (Ueno *et al*., 1989; Durlinger *et al*., 1999). In the ovary, AMH has inhibitory effects on granulosa cell proliferation (Kim *et al*., 1992; Seifer *et al*., 1993), aromatase activity and luteinizing hormone (LH) receptor expression (di Clemente *et al*., 1994). AMH is also an important regulator of the early growth of follicles through inhibitory effects on the recruitment of primordial follicles into the pool of growing follicles (Durlinger *et al*., 1999, 2002a). Furthermore, AMH reduces the sensitivity of large pre-antral follicles and small antral follicles to follicle stimulating hormone (FSH) (Durlinger *et al*., 2001; Gruijters *et al*., 2003). In this way, the AMH secreted by pre-antral and small antral follicles is able to control the recruitment of primordial follicles and the number of follicles that can reach the pre-ovulatory stage (de Vet *et al*., 2002; Gruijters *et al*., 2003; Visser and Themmen, 2005).

In cows, AMH is used as an endocrine marker of the small antral gonadotropin responsive follicle reserve (Rico *et al*., 2009, 2011). Studies carried out in cows with COD have shown plasma and follicular fluid AMH concentrations similar to those of cows with normal ovarian cycles (Monniaux *et al*., 2008; Kitahara *et al*., 2012; El-Sheikh Ali *et al*., 2013).

Considering the multiple functions of AMH in ovarian physiology and taking into account the multiple alterations at ovarian level that exist in the animals with follicular persistence and cystic ovarian disease, we hypothesize that there is an alteration in the expression of this hormone that could affect these processes. The aim of the present study was to examine the ovarian expression and follicular fluid concentrations of AMH in cows with spontaneously arising COD and cows with ovarian follicles with 5, 10 and 15 days of persistence, developed in response to long-term administration of progesterone.

Materials and Methods

Animals

All the procedures were approved by the institutional ethics and security committee of Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, Argentina (protocol numbers 44/10 and 131/12), and are consistent with the 'Guide for the Care and Use of Agricultural Animals in Research and Teaching' (2010). This study was performed in Argentinean Holstein cows with regular oestrous cycles, all of which had calved at least once. For the experimental protocol, the animals were obtained at the end of lactation from local commercial farms and housed outside in an open lot. The cows were fed a diet based on alfalfa pasture, oat or rye grass grazing, supplemented with corn and alfalfa silage, corn grain, soybean expeller and hay, following the recommendations of the Nutrient Requirements of Dairy Cattle (2001). For the spontaneously arising COD group, cows from dairy herds of the milk producing region of Santa Fe, Argentina, were used.

Experimental Model

Ovarian activity was synchronized starting with the procedure commonly referred to as 'G6G' (Bello *et al*., 2006), with some modifications (Díaz *et al*., 2015). Holstein cows with one or more corpora lutea identified by transrectal ovarian ultrasonography were enrolled to start the experiment. The synchronization protocol consisted of two doses of prostaglandin (PG) F2α (0.150 mg D-Cloprostenol, Enzaprost D-C; Biogénesis-Bagó, Argentina) administered 12 h apart on day 0 to induce luteolysis, followed by a dose of gonadotropin-releasing hormone (GnRH; 20 mg buserelin acetate, Gonaxal; Biogénesis-Bagó) 2 days later to stimulate ovulation of the pre-ovulatory follicles present. Six days after the first dose of GnRH, the cows started Ovsynch™ (Pursley *et al*., 1995) with an injection of GnRH (20 mg buserelin acetate, Gonaxal; Biogénesis-Bagó). Seven days later, cows received two doses of PGF2α (0.150 mg D-Cloprostenol, Enzaprost D-C; Biogénesis-Bagó), 12 h apart, to ensure luteolysis (completion of the modified synchronization protocol).

After synchronization, the cows were divided into four groups: control ($n = 10$), P5 (5 days of follicular persistence; $n = 10$), P10 (10 days of follicular persistence; $n = 10$) and P15 (15 days of follicular persistence; $n = 10$). Follicular aspiration was performed in five animals from each group and ovariectomy was carried out in the other five animals (both techniques

are described below). Control cows received no additional hormonal treatment. In a previous study (Díaz *et al.*, 2015) we determined the time of ovulation, defined as day 0 of follicular persistence. On average, ovulation occurred at around 4 days after administration of the first dose of PGF2α (range, 101–106 h). Treated cows (groups P5, P10 and P15) were given a low dose of progesterone until 5, 10 and 15 days, respectively, after the expected day of ovulation (day 0). Progesterone was administered using an intravaginal progesterone releasing device (750 mg of micronized progesterone; Pro-Ciclar P4-Zoovet, Argentina) inserted 1 day after the first PGF2α treatment of Ovsync™ to obtain subluteal concentrations of progesterone (1–2 ng/ml), as described by Díaz *et al.* (2015). Daily ultrasonography and blood sampling were performed in all animals.

The follicular dynamics, changes in steroid secretion and gonadotropin profiles of the same groups of animals have been previously published, thus allowing integration of findings (Díaz *et al.*, 2015).

Spontaneously Arising Cystic Ovarian Disease

Cows with spontaneously arising COD ($n = 10$) were used to obtain whole ovaries by ovariectomy and follicular fluid by follicular aspiration. Animals were diagnosed with COD during the periodic reproductive control by transrectal palpation and confirmed by ultrasonography (Amweg *et al.*, 2013). Cysts were defined as any follicular structure with a diameter equal to or greater than 20 mm and present for 10 days or more, without ovulation or corpus luteum formation, and without uterine tone (Silvia *et al.*, 2002; Bartolomé *et al.*, 2005).

Ovariectomy

Bilateral ovariectomy was performed in control cows, 2 days after completion of the synchronization protocol (48 h after the first PGF2α treatment of Ovsynch™) and on days 5, 10 and 15 of follicular persistence in groups P5, P10 and P15, respectively (Marelli *et al.*, 2014; Díaz *et al.*, 2015). In the group with spontaneously arising COD, ovariectomy was conducted when the veterinarian responsible for the reproductive health of the animals indicated ovariectomy. The ovaries were intended for analysis of protein expression by immunohistochemistry (IHC).

Small samples of ovarian tissues from each group were frozen immediately at −80°C until used in western blotting to determine the specificity of the antibody used in IHC and for other studies.

Follicular Fluid Aspiration

Follicular fluid samples from the control, P5, P10, P15 and spontaneously arising COD groups were obtained using a digital ultrasound system (8300vet Chison) equipped with a microconvex transducer of 5.0 MHz mounted on a transvaginal probe for follicular aspiration (Watanabe Applied Technology Limited, Sao Paulo, Brazil). The follicular fluid was transported to the laboratory, refrigerated on ice and then centrifuged for 10 min at 2,700 g to separate pure follicular fluid from cells. Follicular fluid was stored at −80°C.

The health status of the follicles was confirmed by measuring hormone concentrations in the follicular fluid. Only antral follicles categorized as oestrogen active (17β-oestradiol:progesterone ratio >1) and non-atretic (17β-oestradiol:testosterone ratio >1) were used (data previously published; Díaz *et al.*, 2015).

Tissue Sampling and Follicular Classification

For IHC, the ovaries obtained by ovariectomy from the control, P5, P10, P15 and spontaneously arising COD groups were fixed in 4% buffered formaldehyde for 8–10 h at 25°C and then washed in phosphate buffered saline (PBS). Then, fixed tissues were dehydrated in an ascending series of ethanols, cleared in xylene and embedded in paraffin wax. Sections (5 μm) were mounted on slides previously treated with 2% (v/v) 3-aminopropyltriethoxysilane in acetone (Sigma–Aldrich, St. Louis, Missouri, USA) and stained with haematoxylin and eosin (HE) for preliminary observation of the ovarian structures (Díaz *et al.*, 2015). Follicles were classified into the following groups: primary, small pre-antral, large pre-antral, antral, atretic follicles (Braw-Tal and Yossefi, 1997), persistent follicles (Díaz *et al.*, 2015) and follicular cysts (Silvia *et al.*, 2002). Only follicular cysts and persistent follicles without luteinization and with a complete granulosa cell layer were analyzed.

Immunohistochemistry

IHC was performed with at least five sections of each ovary from each animal. A total of approximately 15 sections were taken for evaluation from each animal (between eight and 15 follicles of each category were evaluated). A streptavidin–biotin immunoperoxidase method was performed as previously described (Matiller *et al.*, 2014). Briefly, after dewaxing, microwave pretreatment (antigen retrieval) was performed by incubating the sections in 0.01 M citrate buffer (pH 6.0). Endogenous peroxidase activity was inhibited with 3% (v/v) H_2O_2 in methanol, and

non-specific binding was blocked with 10% (v/v) normal goat serum in PBS. All sections were incubated with the primary antibody (monoclonal clone 5/6, Serotec MCA2246T, Serotec, Kidlington, UK) at a dilution of 1 in 100, for 18 h at 4°C and then with biotinylated secondary antibody 'ready to use' (CytoScan™ HRP Detection System; Cell Marque, Rocklin, California, USA) for 30 min at room temperature. Labelling was 'visualized' by the CytoScan™ HRP Detection System, and 3,3′-diaminobenzidine (DAB Liquid DAB—Plus Substrate Kit; Invitrogen, Camarillo, California, USA) was used as the chromogen. Finally, the slides were washed in distilled water and counterstained with Mayer's haematoxylin, dehydrated and mounted. Histological sections of bovine ovaries with follicles with proven high expression for AMH were used as positive controls. As negative control and to verify the specificity of labelling, adjacent control sections were subjected to the same immunohistochemical method, replacing the primary antibody with non-immune mouse serum. Additionally, the specificity of the secondary antibody was tested by incubation with anti-human CD45 (clone: PD7/26; Dako, Carpinteria, California, USA), a primary antibody against human antigens with a proven negative reaction to cattle tissues. To exclude the possibility of non-suppressed endogenous peroxidase activity, some sections were incubated with DAB alone. Positive and negative control slides were used for each assay.

Image Analysis

Images were analyzed using Image Pro-Plus 3.0.1 (Media Cybernetics, Silver Spring, Massachusetts, USA). For IHC, images were digitized using a CCD colour video camera (Nikon DS-Fi2, Tokyo, Japan) mounted on a conventional light microscope (Nikon Eclipse *Ni*) and using an objective magnification of ×40. The microscope was prepared for Koehler illumination. This was achieved by recording a reference image of an empty field for the correction of unequal illumination (shading correction) and calibrating the measurement system with a reference slide to determine background threshold values. The reference slides contained a series of tissue sections in which the primary antibodies were replaced by non-immune mouse serum. The positive controls (bovine ovarian follicles of proven positive expression) were used as interassay controls to maximize the levels of accuracy and robustness of the method. The slides were scanned, left to right from the top, and all follicles in the selected categories (between eight and 15 follicles of each category) were analyzed. The percentage of the labelled area was calculated in the granulosa cell layer of the different categories of follicles from ovaries of all groups.

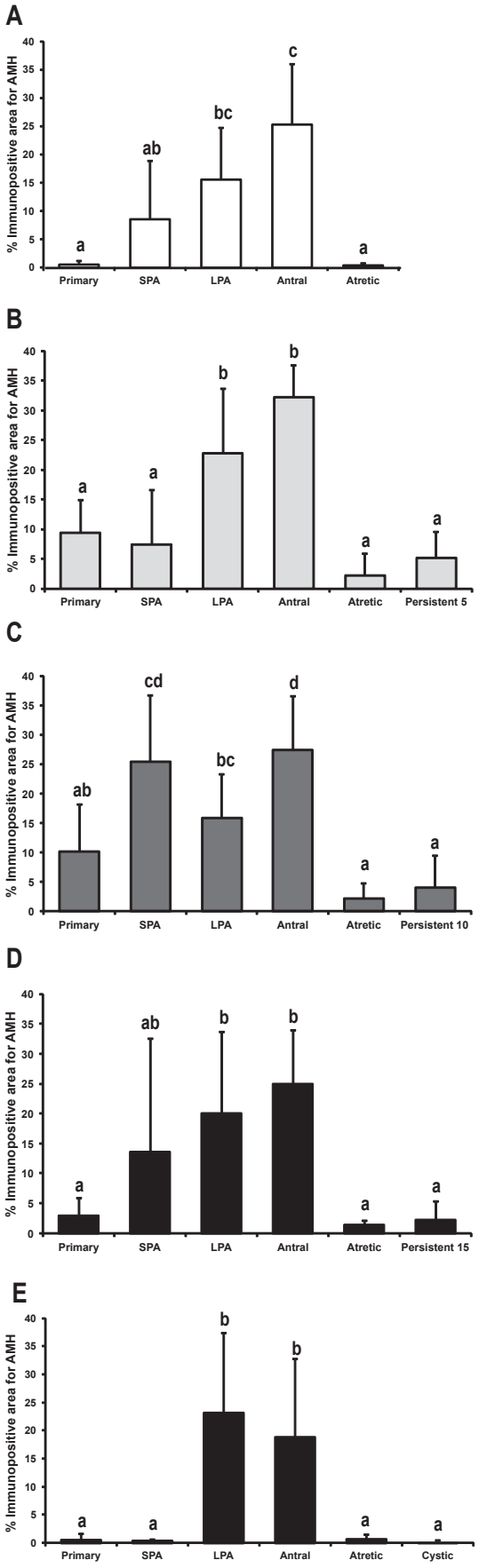
The percentage of the labelled area was calculated as a percentage of the total area evaluated through the colour segmentation analysis, which extracts objects by locating all objects of the specific colour (i.e. brown labelling). The methodological details of image analysis as a valid method for quantification have been described previously (Ortega *et al.*, 2009).

Anti-Müllerian Hormone Concentration in Follicular Fluid

The follicular fluid concentrations of AMH in the control, P5, P10, P15 and spontaneously arising COD groups were quantified using a commercial enzyme-linked immunosorbent assay (ELISA) kit (AMH Gen II, Beckman Coulter, California, USA), following the manufacturer's instructions. Initially, all follicular fluid samples were diluted 10-fold in bovine serum albumin. Follicular fluid samples and standards were placed in duplicate wells of a plate coated with anti-AMH antibody and incubated at room temperature for 2 h. After incubation and washing, anti-AMH detection antibody labelled with biotin was added to each well and incubated. Then, streptavidin—horseradish peroxidase (HRP) was added to the wells and incubation was performed for 30 min. The reaction was 'visualized' by the addition of 3,3′,5,5′-tetramethylbenzidine as chromogen solution. The reaction was stopped with H_2SO_4 and the absorbance measurement was determined at 450 nm with an ELISA reader (MS Microplate Reader, Thermo/Labsystems Inc., Illinois, USA). The detection limit of the kit was 2 pg/ml, and the coefficients of intra- and interassay variation were 8% and 4%, respectively.

Western Blotting

To test the specificity of the antibody used, complete follicular wall composed of granulosa and theca cells of tertiary follicles and follicular cysts were homogenized in a radio-immunoprecipitation assay 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—Aldrich Corp.), 0.1 M PBS and a protease inhibitor cocktail (Complete Mini Protease Inhibitor Cocktail Tablets, Roche, Mannheim, Germany). Follicular 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 sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (15% resolving gel) under reducing



conditions, transferred onto nitrocellulose membranes (GE-Healthcare, Buckinghamshire, UK), blocked for 5 h in 5% non-fat milk in Tris-buffered saline containing 0.05% Tween-20 (Sigma–Aldrich Corp.), and then incubated overnight at 4°C with the specific primary antibody used in IHC (clone 5/6, Serotec MCA2246T). Following washing, membranes were treated with a secondary peroxidase-conjugated antibody for 1 h (goat anti-mouse IgG HRP, SC-2005, Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA). The immunopositive bands were detected by chemiluminescence using the ECL-plus system (GE-Healthcare) on hyperfilm-ECL film (GE-Healthcare).

Statistics

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. SPSS 11.0 for Windows (SPSS Inc., Chicago, Illinois, USA) was used to perform the statistical tests. The distribution of data was tested for normality using the Kolmogorov–Smirnov test. Differences between two groups of data were detected by non-paired two-tailed Student's t-test. The differences between more than two groups of data were assessed by one-way ANOVA, followed by Duncan's multiple range tests. $P < 0.05$ was considered significant. Results are expressed as mean ± standard deviation (SD).

Results

Anti-Müllerian Hormone Expression and Localization

AMH was expressed in the granulosa cells of all follicles in all of the experimental groups. In contrast, AMH expression in theca interna cells was extremely low and could not be analyzed quantitatively.

In granulosa cells, primary, atretic (all groups), persistent (P5, P10 and P15 groups) and cystic follicles (spontaneously arising COD group) had the lowest AMH expression throughout folliculogenesis (Fig. 1) ($P < 0.05$).

Fig. 1. Relative AMH protein expression throughout folliculogenesis (measured as percentage of immunolabelled area) in granulosa cells of different follicular categories from the control (A), P5 (B), P10 (C), P15 (D) and spontaneously arising COD (E) groups. Values represent mean ± SD. Bars with different letters are different ($P < 0.05$). SPA, small pre-antral follicle; LPA, large pre-antral follicle.

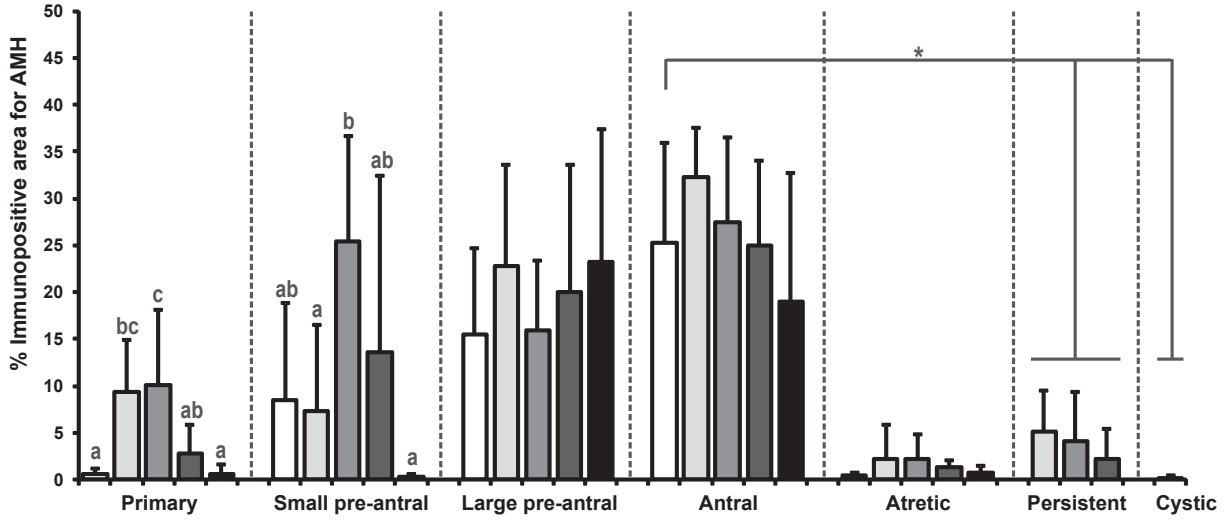


Fig. 2. AMH protein expression in different follicular categories from the control, P5, P10, P15 and spontaneously arising COD groups. Relative protein expression (measured as percentage of immunolabelled area) of AMH in granulosa cells of primary, small pre-antral, large pre-antral, antral and atretic follicles (all groups), persistent follicles (groups P5, P10 and P15) and cystic follicles (spontaneously arising COD group). Bars with different colours indicate the different groups: control ovaries (open bars), P5 (light grey bars), P10 (moderate grey bars), P15 (dark grey bars) and COD (black bars). Values represent mean ± SD. Bars with different letters within a category are significantly different ($P < 0.05$). An asterisk indicates differences between spontaneously arising cysts and persistent follicles with antral follicles from the control group ($P < 0.05$).

In the control group, AMH expression showed a gradual increase throughout folliculogenesis and the expression in primary, small pre-antral and atretic follicles was lower than that in antral follicles. Moreover, the expression in large pre-antral follicles was higher than that in primary and atretic follicles ($P < 0.05$) (Fig. 1A). In group P5, AMH expression was higher in follicles in advanced stages of development (i.e. large pre-antral and antral follicles) than in primary, small pre-antral, atretic and persistent follicles ($P < 0.05$) (Fig. 1B). In group P10, AMH expression was lower in primary, large pre-antral, atretic and persistent follicles than in antral follicles ($P < 0.05$) (Fig. 1C). In group P15, AMH expression was higher in large pre-antral and antral follicles than in primary, atretic and persistent follicles, without differences with small pre-antral follicles ($P < 0.05$) (Fig. 1D).

In the group with spontaneously arising COD, AMH expression throughout folliculogenesis had a pattern similar to that of the other groups analyzed, being higher in large pre-antral and antral follicles than in primary, small pre-antral, atretic and cystic follicles ($P < 0.05$) (Fig. 1E).

In the analysis between groups, the primary follicles from the control and spontaneously arising COD groups had lower AMH expression than those from groups P5 and P10 ($P < 0.05$). Small pre-antral follicles from group P10 had higher levels than those from the P5 and spontaneously arising COD groups ($P < 0.05$). In the large pre-antral and antral category, no differences were detected between the groups evaluated. Likewise, the analysis of atretic and persistent follicles showed a very low level of immunohistochemically labelled AMH. Furthermore, in these two categories, no differences were found between groups (Fig. 2).

Comparison between antral follicles from the control group (as a reference structure) and persistent and cystic follicles showed lower expression in persistent follicles of all groups and in cystic follicles ($P < 0.05$) (Fig. 2).

Representative images of AMH labelling in small pre-antral, large pre-antral, antral and atretic follicles (all groups), persistent follicles (groups P5, P10 and P15) and cysts (spontaneously arising COD group) are shown in Fig. 3.

In western blotting analysis, AMH was detected as a single intense band at 30 kDa (Fig. 3).

Concentration of Anti-Müllerian Hormone in Follicular Fluid

AMH concentration in follicular fluid was lowest in the control and spontaneously arising COD groups ($P < 0.05$) and highest in groups P10 and P15. In this sense, the concentrations found at 10 and 15 days of follicular persistence were higher than those found in the spontaneously arising COD group ($P < 0.05$). Furthermore, the concentrations at 10 days of persistence were higher than those found in the control group ($P < 0.05$) (Fig. 4).

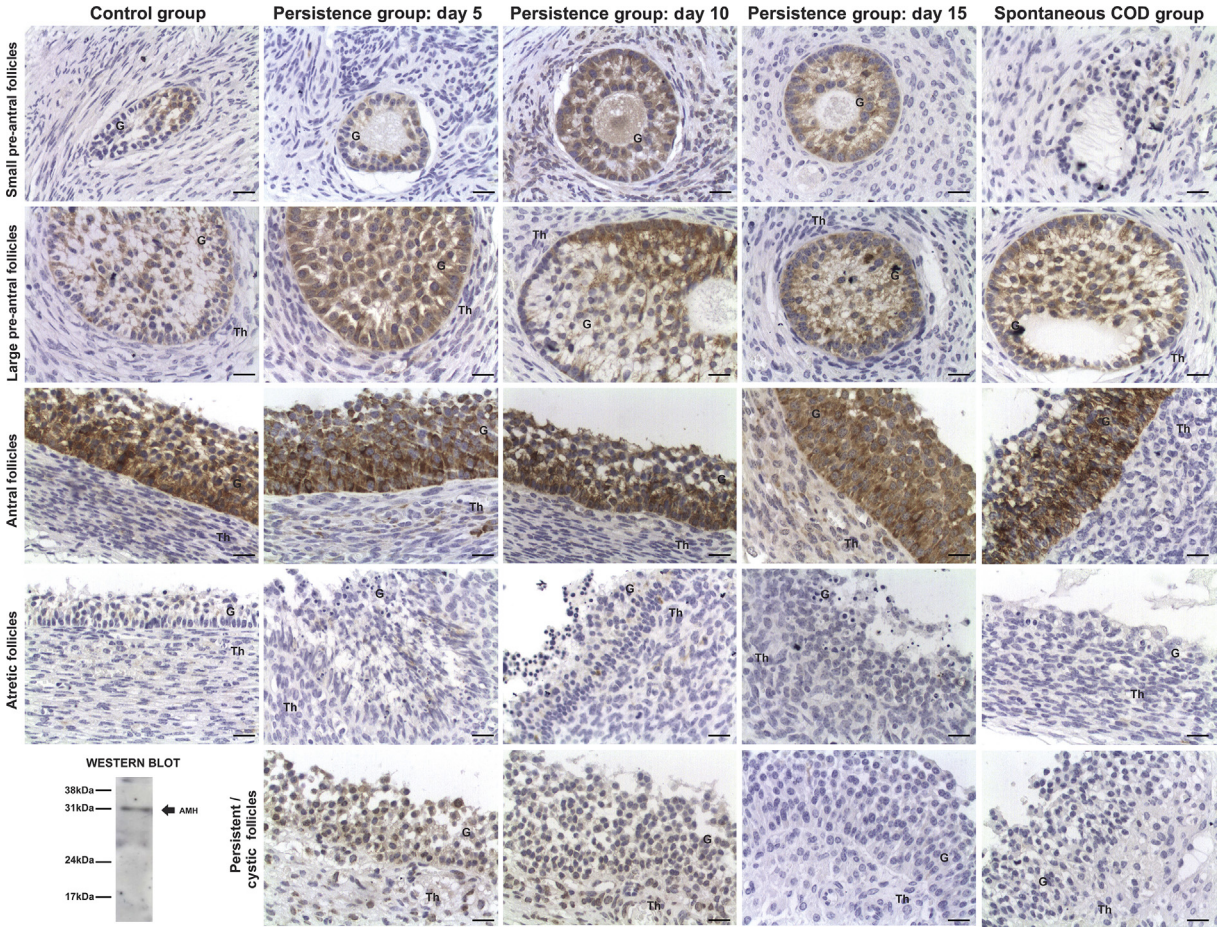


Fig. 3. Representative images of AMH immunolabelling in small pre-antral, large pre-antral, antral and atretic follicles (all groups), persistent follicles (groups P5, P10 and P15) and cysts (spontaneously arising COD group). Note that granulosa cells, atretic, persistent and cystic follicles showed the lowest AMH expression throughout the experimental protocol. Granulosa (G), theca interna (Th). Bars, 20 μm. A representative western blot is shown in the lower left corner. Black arrow indicates the AMH molecular weight of 30 kDa.

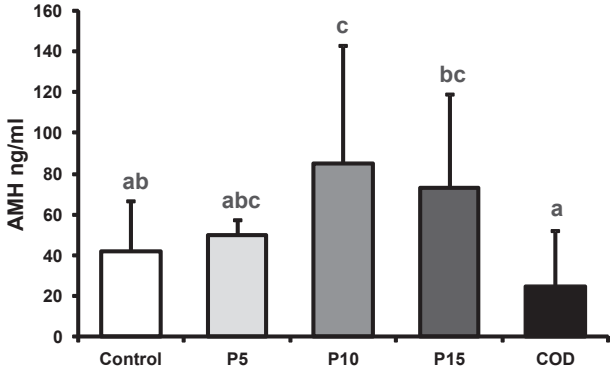


Fig. 4. AMH concentration in follicular fluid of pre-ovulatory follicles (control group), persistent follicles (groups P5, P10 and P15) and cysts (spontaneously arising COD group). Bars with different colours (grey scale) indicate different groups. Values represent mean ± SD. Bars with different letters are significantly different ($P < 0.05$).

Discussion

This study is the first to evaluate AMH expression at different times of follicular persistence associated with COD and to compare it with that in spontaneously arising COD. We found AMH expression in granulosa cells in all follicular categories of each group studied. AMH expression in pre-antral and antral follicles was significantly higher than that in the persistent follicles from groups P5, P10 and P15, and in atretic follicles from all groups (control, persistence and spontaneously arising COD groups). This agrees with the findings by Rico *et al.* (2011), who detected AMH expression in pre-antral and antral follicles and cumulus oophorus cells, without differences in the intensity of immunohistochemically labelling between the different groups studied. In the same way, other authors have observed that AMH expression in the granulosa decreases as the state of follicular atresia advances (Knight and Glister, 2006; La

Marca and Volpe, 2006; Rico *et al.*, 2009, 2011). Likewise, we observed that AMH expression was significantly lower in granulosa cells from cystic and persistent follicles than in antral follicles from the control group. No differences were observed between spontaneously arising cysts and persistent follicles. These results, added to those obtained in previous studies (Díaz *et al.*, 2015), support the utility of the experimental protocol of follicular persistence and demonstrate that it is a good model that effectively replicates what happens in spontaneously arising COD. Moreover, we observed a higher concentration of AMH in follicular fluid from persistent follicles of 10 and 15 days compared with spontaneously arising follicular cysts. This partially agrees with the results of Monniaux *et al.* (2008), who reported that the AMH concentration in follicular fluid and mRNA expression in granulosa cells showed no differences from those in large antral follicles and cysts. Similarly, El-Sheikh Ali *et al.* (2013) found no differences in the plasma concentrations of AMH in samples from animals with spontaneously arising COD and samples from animals with normal oestrous cycles. This could indicate that changes in the secretion of AMH occur progressively as the time of persistence progresses.

Although some studies have established that intrafollicular and plasma AMH concentrations cannot be used as biomarkers to determine the presence of COD in cows (Monniaux *et al.*, 2008; Kitahara *et al.*, 2012; El-Sheikh Ali *et al.*, 2013), they did not analyze the follicular fluid levels during the early stage of COD development. As indicated above, in the present study, the persistent follicles of the progesterone-induced model showed high concentrations of AMH at 10 and 15 days of persistence. In this sense, Polat *et al.* (2015) showed that, in cows, the follicular fluid concentration of AMH found in cysts was not significantly different from that found in regressing antral follicles developed in the presence of a corpus luteum. However, these authors also showed that the AMH concentration in pre-ovulatory follicles was significantly lower than that in cysts. Furthermore, in concordance with our findings, these authors found that AMH protein expression was very weak both in follicular cysts and in regressing antral follicles at di-oestrus (Polat *et al.*, 2015). Nevertheless, it should be clarified that these authors studied type 2 cysts (Braw-Tal *et al.*, 2009), which have high progesterone concentrations. In this sense, we have previously found that although persistent follicles at 10 and 15 days of persistence have low progesterone concentrations, they have high levels of 17-hydroxyprogesterone (17OHP4) in follicular fluid and serum (Díaz *et al.*, 2015), which could have a response similar to that of progesterone, due to its ability to act on the same progesterone receptor (Blackmore *et al.*, 1990; Ashley *et al.*, 2006; Manuck *et al.*, 2011). Therefore, 17OHP4 would be acting in a manner similar to that of the progesterone concentrations existing in type 2 cysts.

On the other hand, it should be considered that bone morphogenetic proteins-6 and -4 (BMP-6 and BMP-4) enhance AMH secretion in follicles of 5−10 mm diameter and increase its mRNA expression in follicles of 3−5 mm and 5−10 mm in diameter (Rico *et al.*, 2011). These results can be related to our previous findings, in which we observed an increase in BMP-4 and BMP-6 expression in the granulosa and theca cells of various follicular categories, including persistent follicles of the follicular persistence groups in relation to controls (Díaz *et al.*, 2016). These stimuli by local factors such as BMPs could partially control the increase in AMH follicular fluid concentration. It is currently accepted that several post-transcriptional mechanisms, such as the secretion rate and protein binding to the extracellular matrix, may regulate the accumulation of growth factors in the follicular antrum (Gumienny and Padgett, 2002; Monniaux *et al.*, 2008).

AMH exerts a negative effect on the primordial to primary follicle transition and consequently on pre-antral follicle development (Knight and Glister, 2006). Durlinger *et al.* (2001) showed that AMH inhibits the FSH-dependent growth of antral follicles. The high concentrations of AMH found in follicular fluid from follicles with 10 and 15 days of persistence could exert a negative effect on the early stage of follicular development, preventing their development to more advanced stages (i.e. pre-antral follicles). All of these events lead to a fall in the quantity of new follicular waves or to an alteration in the capacity to respond to a pituitary gonadotropin stimulus. Additionally, AMH inhibits the aromatase (CYP19A1) enzyme and the FSH-induced LH receptor in antral follicle granulosa cells (di Clemente *et al.*, 1994; Monniaux *et al.*, 2008). Through these functions, AMH reduces the FSH response of pre-antral and small antral follicles and therefore exerts a negative role in the cyclic recruitment and selection process of the dominant follicle (Durlinger *et al.*, 2002a, 2002b; Visser and Themmen, 2005; Knight and Glister, 2006). Additionally, we have previously found that follicles with 15 days of persistence show a decrease in follicular fluid oestrogen concentrations in association with an increase in testosterone levels. These findings can be due to a loss of granulosa cell layers during follicular persistence (Díaz *et al.*, 2015) and/or to the inhibition of CYP19A1 induced by AMH (di Clemente *et al.*, 1994; Monniaux *et al.*, 2008).

In summary, the results presented here lead us to conclude that the expression and concentration of AMH is clearly altered in the course of follicular persistence and in developed COD. This can lead to altered ovarian function, contributing to the follicular persistence and endocrine/paracrine changes found in cattle with COD.

Acknowledgments

This study was supported by a grant from the Argentine National Agency for the Promotion of Science and Technology (ANPCyT) (PICT 2010-2315, 2011-1274 and 2013-2279). We are grateful to the staff of the Hospital de Salud Animal of the Facultad de Ciencias Veterinarias de la Universidad Nacional del Litoral for animal care and help with the generation of experimental animals and collection of samples. We also acknowledge the staff members of the Laboratorio de Biología Celular y Molecular Aplicada (ICiVet-Litoral, UNL-CONICET) for technical support in processing of slides. We thank J. Bertoli and F. Barberis for assistance with the animal care and Biogénesis-Bagó and Zoovet Laboratories for providing the drugs used in the protocols. The first two authors contributed equally to this work.

Conflict of Interest Statement

The authors declare no potential conflicts of interest related to publication of this research.

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[*Received, August 23rd, 2017*
Accepted, October 21st, 2017]