



Hemodynamic changes detected by Doppler ultrasonography in the ovaries of cattle during early development of cystic ovarian disease

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ARTICLE INFO

Keywords:

Cystic ovarian disease
Doppler ultrasonography
Follicular persistence
Bovine

ABSTRACT

A common reproductive disease in dairy cattle is Cystic Ovarian Disease. To study its development, there was use of an experimental model of follicular persistence to detect hemodynamic changes occurring in ovaries by using Doppler ultrasonography. After estrous synchronization, control cows received no additional treatment and were evaluated at proestrus (CG), whereas treated cows (PG) received sub-luteal doses of progesterone for 15 days and were evaluated at proestrus, and after 0, 5, 10 and 15 days of follicular persistence. Spectral Doppler was used to evaluate blood flow in the ovarian artery, and power Doppler for evaluation of blood flow in the ovarian parenchyma and follicular wall of persistent and dominant preovulatory follicles. Findings using power Doppler signals indicated there were no differences between groups in the parenchyma of both right ($P = 0.455$) and left ($P = 0.762$) ovaries. In contrast, power Doppler signals of blood flow were less in walls of persistent follicles from day 0 to 15 when there was follicular persistence than in dominant follicles of the CG ($P < 0.001$). Blood flow in ovarian arteries was less ($P < 0.05$) in diastolic velocity and time averaged maximum velocity in all PG groups than in the CG. Peak systolic velocity was less ($P < 0.05$) in all PG than in the CG, with the exception of P15 ($P > 0.05$). These findings indicate there are marked changes in blood irrigation area of walls of persistent follicles during the 15 days of follicular persistence.

1. Introduction

Angiogenesis refers to the formation of new blood vessels, or neovascularization, and is essential for normal tissue growth and development (Folkman and Klagsbrun, 1987; Klagsbrun and D'Amore, 1991). This process, which begins with capillary proliferation and culminates in the formation of a new microcirculatory bed composed of arterioles, capillaries and venules, has an essential function in the female reproductive system. The female reproductive organs have periodic growth and regression stages, accompanied by notable changes in blood flow rates (Redmer and Reynolds, 1996). In the ovary, the follicles and corpora lutea receive blood supply through a complex network of blood vessels. During the reproductive cycle, the ovarian vasculature undergoes important changes in size and morphology. These functional and structural changes occur mainly in the microvasculature of the luteo-

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follicular complex and have important functions in folliculogenesis, ovarian hormone production and ovulation (Jiang et al., 2003). Angiogenesis is individually regulated within each follicle and its development is accompanied by qualitative and quantitative changes in the microvascular bed (Hazzard and Stouffer, 2000; Plendl, 2000; Fraser, 2006), which, among other things, allows a greater uptake of gonadotropins (McNatty et al., 1981; Zeleznik et al., 1981; Reynolds and Redmer, 1998). After the luteinizing hormone (LH) preovulatory surge release from the anterior pituitary and prior to ovulation, there is an increase in the vasodilation, with major vascular permeability, hyperemia and an increase in the ovarian blood flow (Abulafia and Sherer, 2000; Acosta et al., 2003).

For more than 15 years, blood flow studies in the reproductive organs of cattle have been performed using color Doppler ultrasonography (Bollwein et al., 2016). This technique allows for quantifying follicular and luteal blood flow by measuring the colored area in relation to the total area of these structures detected on B-mode images or in power mode by using a computer image analysis software (Herzog and Bollwein, 2007; Bollwein et al., 2016). In cows, Doppler ultrasonics has been used to study changes that occur after administration of a gonadotropin releasing hormone (GnRH) analog. With this GnRH treatment, the blood flow to the ovulatory follicle increases simultaneously with an acute increase in LH concentration (Acosta et al., 2003).

Both follicular growth and follicular atresia are regulated by the extent and type of vascular development (Hazzard and Stouffer, 2000) and an alteration in this process can result either in a disruption of the physiological state or in the occurrence of pathological conditions (Abulafia and Sherer, 2000). Some pathological conditions such as Cystic Ovarian Disease (COD), one of the most frequent reproductive diseases in dairy cattle, can only be studied retrospectively, when the cyst is already formed and has undergone numerous changes. The incidence of COD in dairy herds varies from 5% to 30% (revised in Ortega et al., 2016). This condition results in significant economic losses in the dairy industry by decreasing artificial insemination pregnancy rates, increasing calving-to-conception and inter-calving intervals and, if the condition persists, the animals may be culled from the herd (reviewed in Ortega et al., 2016). The use of experimental models may allow for elucidation of the changes that occur in anovulatory follicles during the developmental stages previous to the formation of cysts, offering an opportunity to understand the pathogenesis of diseases such as COD (Díaz et al., 2015; Ortega et al., 2016). There has been previous development of an experimental model to induce persistent follicles based on the long-term administration of progesterone in amounts that lead to sub-luteal concentrations in blood (Díaz et al., 2015, 2016; Stassi et al., 2017; Díaz et al., 2018). With this model, the endocrine profile, growth dynamics, and histological characteristics of persistent follicles are analogous to those of spontaneous-developed cysts indicating there is a local function of the sub luteal concentration of progesterone in the pathogenesis of COD and in the regulatory mechanisms of ovarian function (Díaz et al., 2015, 2016; Stassi et al., 2017). Considering that all these mechanisms directly affect ovarian remodeling and the processes that leads to ovulation, and that follicular persistence has been described as a failure in this process, the objective of the present study was to evaluate the hemodynamic changes using color Doppler ultrasonography that occur in the ovary during the development of persistent ovarian follicles in a model of follicular persistence induced with exogenous progesterone in dairy cattle.

2. Materials and methods

2.1. Animals

All the procedures were approved by the Institutional Ethics and Security Committee of the Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, Argentina (protocol no. 298/16), and are consistent with the "Guide for the Care and Use of Agricultural Animals in Research and Teaching, Third Edition" (Federation of Animal Science Societies, 2010). This study was performed in non-lactating Holstein cows ($n = 18$) having regular estrous cycles. Cows were housed outside in an open lot, except during ultrasonic examinations (when they were moved to a stanchion barn). The cows were fed with a diet based on alfalfa pasture, corn silage and commercial balanced food, following the recommendations of the [Nutrient Requirements of Dairy Cattle \(2001\)](#).

2.2. Induction of follicular persistence by progesterone administration

Cows that did not have reproductive or infectious problems (such as mastitis or metritis) were used to conduct this study. Rectal palpation and ultrasonography were performed to evaluate the functional changes occurring during the estrous cycle, selecting those animals with one or more active corpora lutea or growing follicles as parameters of normal ovarian functions. The stage of the estrous cycle of cows was synchronized using the "G6G protocol" (Pursley et al., 1995; Bello et al., 2006) with some modifications (Díaz et al., 2015). The estrous synchronization protocol consisted of two doses of prostaglandin F_{2α} (PGF_{2α}; 150 μg D + cloprostenol; Enzaprost DC, Biogénesis Bagó, Argentina) administered 12 h apart on day 0 to induce luteolysis, followed by a dose of GnRH (20 μg buserelein acetate; Gonaxal, Biogénesis Bagó) 2 days later to stimulate ovulation of the dominant preovulatory follicles present. After 6 days from the time of the first GnRH dose, the Ovsynch protocol was initiated with another injection of GnRH. After 7 days, cows received two doses of PGF_{2α}, 12 h apart, to ensure luteolysis (completion of the modified estrous synchronization protocol). After estrous synchronization, cows were divided into two groups: pre-ovulatory control group (CG; $n = 10$) and treated (or persistence) groups (PG; $n = 8$). Control cows received no additional hormonal treatment. On average, ovulation in the control cows occurred around 4 days after administration of the first PGF_{2α} dose (range 101–106 h). To prevent ovulation of treated cows, one day after the first PGF_{2α} injection of Ovsynch, an intravaginal progesterone releasing device (750 mg micronized progesterone; Pro Ciclar P4 Zoovet, Argentina) was inserted to obtain sub-luteal concentrations of progesterone in blood (1–2 ng/mL), as previously described (Díaz et al., 2015). The intravaginal progesterone releasing device remained in place for 8 days in all treated cows. A new intravaginal progesterone releasing device was inserted 1 day before removal of the first one (day 4 of persistence) to maintain a more

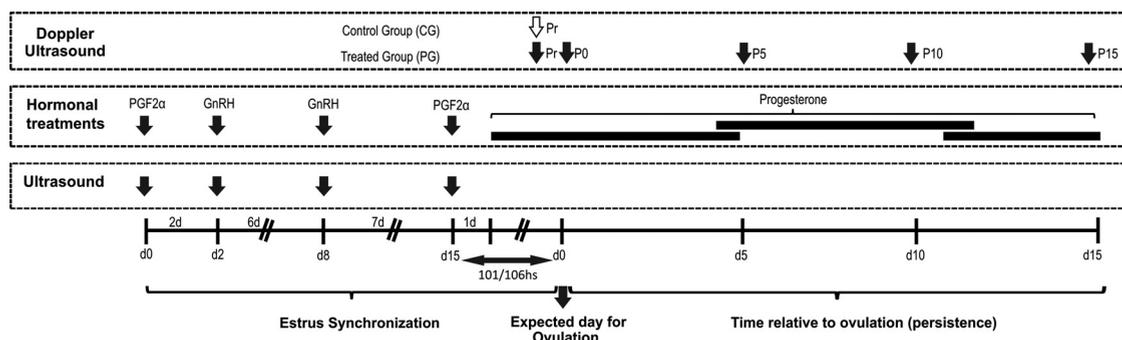


Fig. 1. Experimental design for the induction of follicular persistence. Holstein cows were synchronized and received an intravaginal progesterone-releasing device to induce follicular persistence. Follicular changes as a result of estrous synchronization were monitored using ultrasonography. Hemodynamic changes were evaluated using Doppler ultrasonography at proestrus (Pr), and after 0 (P0), 5 (P5), 10 (P10) and 15 (P15) days of follicular persistence. CG: control group, PG: treated (persistence) group. Black bars: time of permanence of each intravaginal progesterone releasing device.

consistent concentration of progesterone throughout the treatment period. A third intravaginal progesterone releasing device was inserted on day 11 of the persistence phase of the dominant follicle, 1 day before removal of the second one (Fig. 1). Successful induction of progesterone-induced follicular persistence was confirmed by ovarian morphology and hormone concentrations (for more details, see Díaz et al., 2015).

2.3. Ovarian ultrasonography

Animals in the CG and PG were evaluated using Doppler ultrasonography at proestrus, and in PG on the expected day of ovulation (P0), after 5 (P5), 10 (P10) and 15 (P15) days of follicular persistence (Fig. 1). To monitor the changes in the estrous cycle, there were scheduled ultrasonography assessments using portable color Doppler equipment (Mindray Z6 Vet, China) with a 5.0 to 10.0 MHz linear array trans-rectal transducer (75L50EAV, Mindray). The real time B-mode monitoring occurred using the procedures described by Sirois and Fortune (1988). The effectiveness of the estrous synchronization protocol was monitored using ultrasonography on days 0, 2, 8, and 15 (Fig. 1). Furthermore, the spectral Doppler (PW) mode was used to evaluate the blood flow in the ovarian artery, whereas the power Doppler mode was used to evaluate the blood flow intensity in the ovarian parenchyma and the walls of persistent and dominant follicles (Ginther, 2007; Herzog and Bollwein, 2007; Ginther et al., 2014; Bollwein et al., 2016). To conduct these procedures, the ultrasonic equipment was calibrated at the minimal blood flow velocity available on the instrument (5 cm/s) and a frequency of 4.4 MHz in PW mode and 5.3 MHz in power mode, and a low gain setting was used to minimize artifacts (Ginther et al., 2014). To determine possible changes in blood flow velocities and in Doppler indices in PW mode, a sample gate cursor (0.5 mm wide) was placed in a specific artery (most prominent blood flow color spot) located within 1 cm of the base of the ovary. The ultrasonic software was then used to calculate the values for the following variables: systolic/diastolic ratio (S/D), resistance index (RI), pulsatility index (PI), peak systolic velocity (PS), end diastolic velocity (ED), and time averaged maximum velocity (TAMV). Videos from each ovary were stored for image analysis and measurements of ovarian structures. Furthermore, to perform a safe technique and minimize artifacts by the animal movements, all cows were sedated with 6 mg of xylazine hydrochloride (Xilazina 2%, Over, Argentina), injected subcutaneously, and received a caudal epidural anesthesia at the intercocygeal space (Co1-Co2), with 100 mg of lidocaine hydrochloride (Lidocaína 2%, Over) (Ginther et al., 2014).

2.4. Image analysis

The blood perfusion on the ovarian parenchyma and in the walls of dominant preovulatory control and persistent follicles was semi-quantitatively evaluated as described by Pancarci et al. (2011) and Bollwein et al. (2016), with some modifications. The entire cross sectional area of the ovaries was visible within the power Doppler sample box (Fig. 2). Ten images without flash artifacts and with a colored area were selected and stored from video sequences of 1 min maximum duration (Pancarci et al., 2011). Ultrasonic videos were exported in an AVI format to a computer and a total of ten representative ultrasonographic images from different sections of each ovary and follicular walls were stored in a JPG format for image analysis. On the digitized ultrasonographic images, the region of interest was defined by the examiner and the colored area in relation to the total area of the tissue was measured using the computer software Image Pro Plus 3.0.1 (Media Cybernetics, Silver Spring, MA, USA). The percentage of the blood perfusion area was calculated as a percentage of the total area evaluated using color segmentation analysis, which extracts objects by locating all objects of a specific color. The mean values of the ten images were used for the evaluation of the ovarian and follicular wall blood flow.

2.5. Statistical analysis

All the analyses were performed using SPSS 11.0 for Windows (SPSS Inc., Chicago, IL, USA). For all analyses, cow was considered

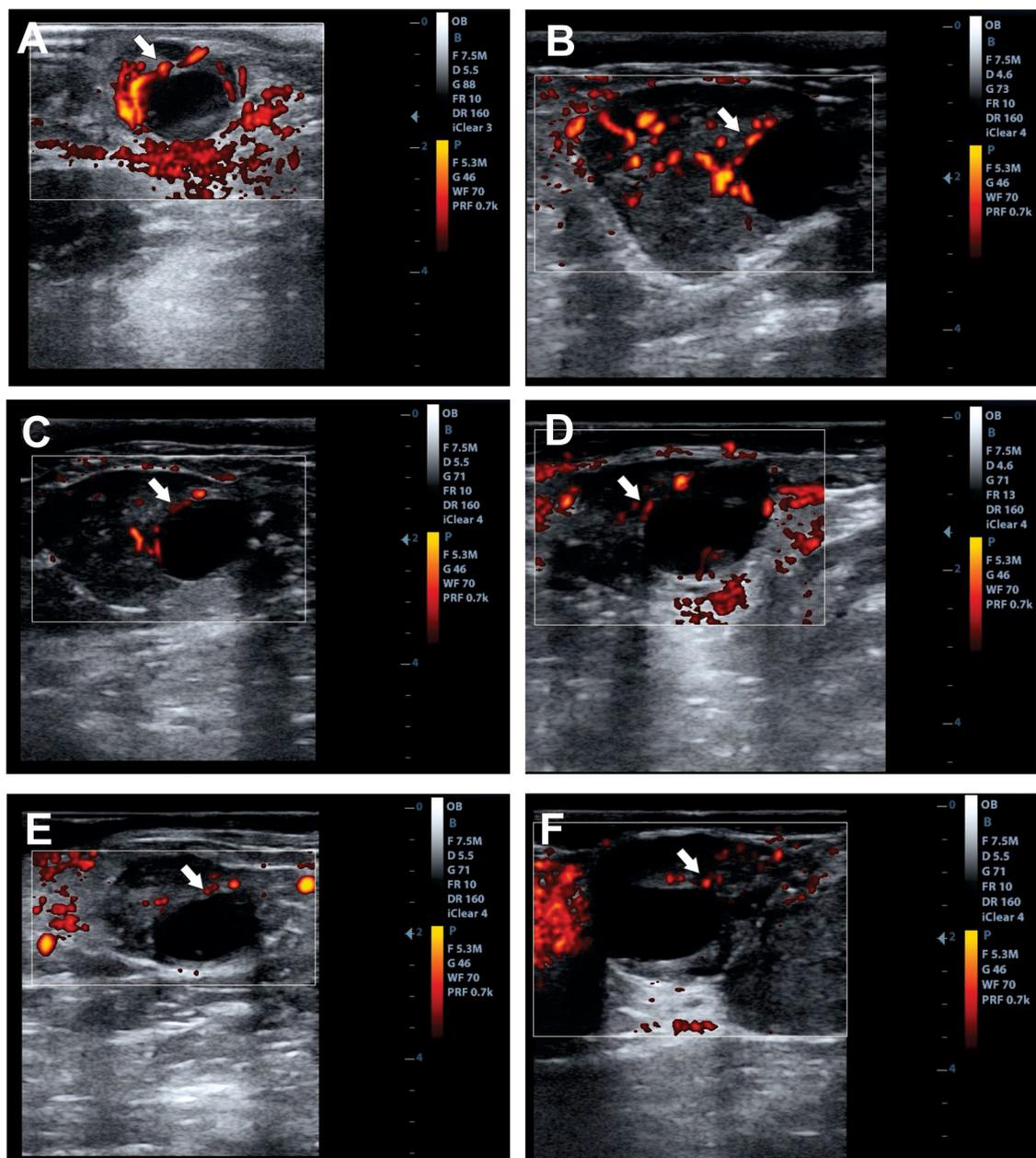


Fig. 2. Selected power Doppler pictures showing the presence of positive signals of blood flow intensity in control cows at proestrus (A) and treated cows at proestrus (B) and after 0, 5, 10 and 15 days of follicular persistence (C, D, E and F, respectively). Note the decrease of power Doppler blood flow signals from the expected day of ovulation (C) until 15 days of follicular persistence (C, D, E and F). White arrows show the blood flow intensity detected by the power Doppler mode in the follicular wall.

the experimental unit. Data collected from power Doppler images were used to evaluate the blood irrigation area (percentage of a structure or tissue with color-Doppler signals for blood flow; [Ginther, 2007](#)) in the ovarian parenchyma and in the follicular wall of the dominant preovulatory/persistent follicles between treated (PG) and control (CG) cows. Data from spectral Doppler assessments was used to evaluate the blood flow velocities and Doppler indices in the ovarian artery ipsilateral to the dominant preovulatory/persistent follicles between cows of the PG and CG. The distribution of data was assessed for normality using the Kolmogorov–Smirnov test. For quantitative outcomes (blood irrigation area and blood flow velocities) and for semi-quantitative outcomes (Doppler indices), one-way analysis of variance (ANOVA) was performed to observe differences between groups, followed by Duncan’s multiple range tests. The $P < 0.05$ and lesser values were considered significant. Results are expressed as mean \pm standard error of the mean (SEM).

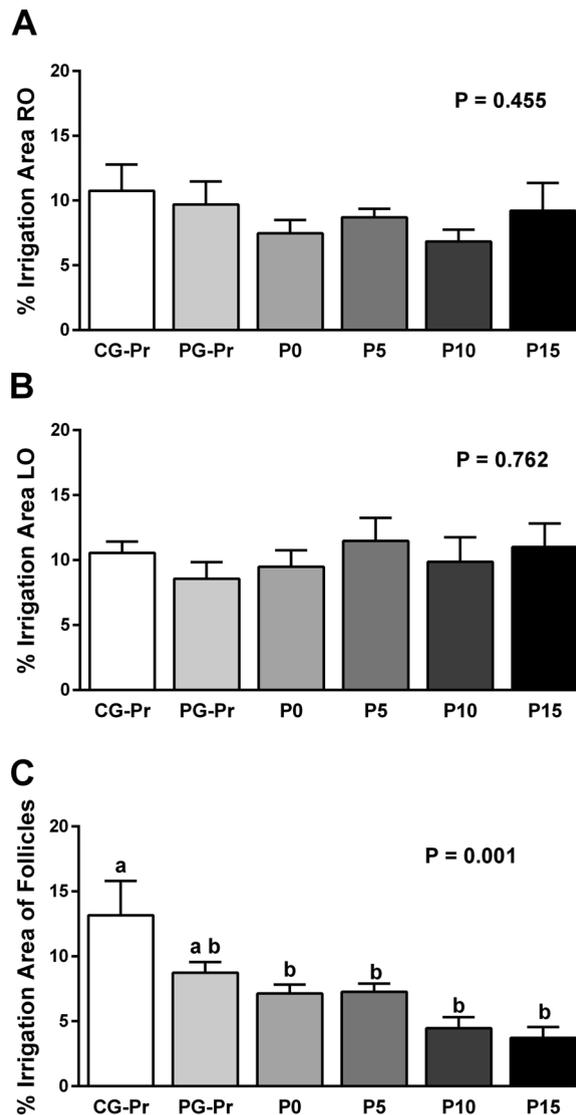


Fig. 3. Percentage of blood irrigation area (power Doppler signals) in the parenchyma of the right (RO) and left (LO) ovaries (A and B, respectively) and in the wall of dominant preovulatory or persistent follicles (C). Dominant preovulatory follicle of control group (CG-Pr, white bars) and persistence group: at proestrus (PG-Pr), on the expected day of ovulation (P0), after 5 days of follicular persistence (P5), after 10 days of follicular persistence (P10) and after 15 days of follicular persistence (P15) (gray bars). Values represent mean \pm SEM. Bars with different letters are different ($P < 0.05$).

3. Results

3.1. Blood perfusion in the ovarian parenchyma

The percentage of the blood irrigation area in both the right and left ovarian parenchyma was evaluated at proestrus in the CG and PG and during follicular persistence at 0, 5, 10 and 15 days of persistence in the PG. The semi-quantitative evaluation of blood flow (power Doppler signals) in the parenchyma could be assessed in all ovaries where evaluations were attempted. There was an obvious presence of a preovulatory follicle at proestrus in the CG and PG (Fig. 2A and B, respectively) and there were one or two regressing corpora lutea during proestrus in the CG, PG and in the PG at P0 (Fig. 2B) or there was a persistent follicle in the PG (Fig. 2C–F). There were no differences in the percentage of the blood irrigation among the different stages of follicular persistence when compared with the values at proestrus in the CG in both the right ($P = 0.455$) and left ($P = 0.762$) ovaries (Fig. 3A and B, respectively).

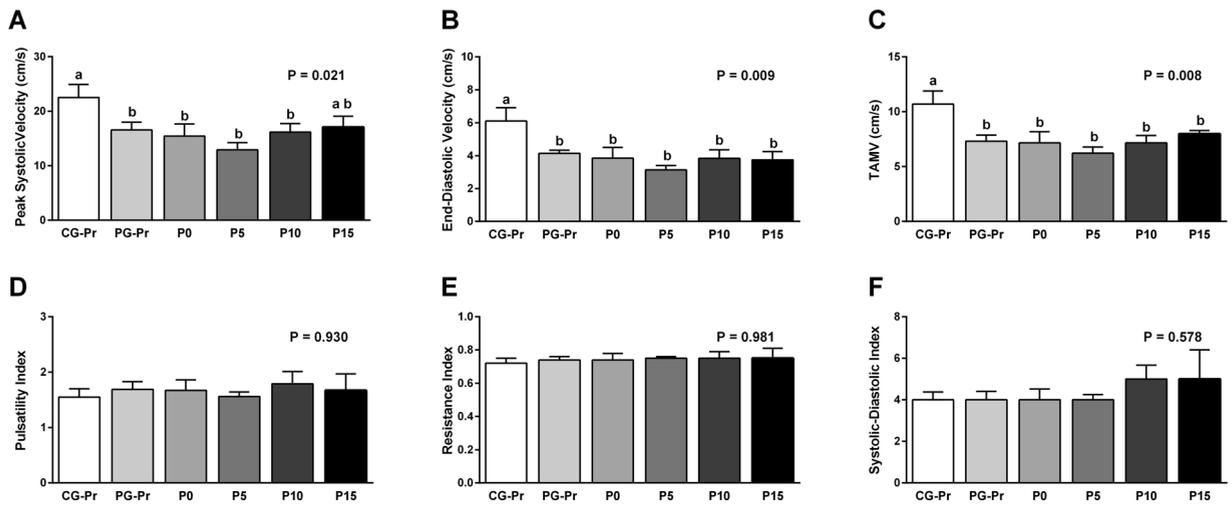


Fig. 4. Spectral Doppler blood flow velocity variables: peak systolic velocity (A), end diastolic velocity (B) and time averaged maximum velocity (TAMV) (C), and spectral Doppler indices: pulsatility index (D), systolic/diastolic ratio (F) and resistance index (E) for the blood flow in the ovarian artery ipsilateral to the dominant preovulatory/persistent follicles of control group (CG-Pr, white bars) and persistence group (PG): at proestrus (PG-Pr), on the expected day of ovulation (P0), after 5 days of follicular persistence (P5), after 10 days of follicular persistence (P10) and after 15 days of follicular persistence (P15) (gray bars). Values represent mean \pm SEM. Bars with different letters are different ($P < 0.05$).

3.2. Blood perfusion in persistent and dominant preovulatory follicles

The power Doppler signals of blood flow in the wall of dominant preovulatory/persistent follicles was evaluated during proestrus in the CG and in all stages of follicular persistence in the PG. The presence of positive signals of blood flow was observed in proestrus in the CG and in all stages of follicular persistence in the PG (Fig. 2). The percentage of the blood irrigation area in the wall of persistent follicles from day 0 to day 15 of follicular persistence was less ($P = 0.001$) than those observed in the wall of preovulatory follicles from the CG (Fig. 3C). Blood flow signals in the wall of persistent follicles decreased from the expected day of ovulation (P0) and remained at these lesser values until day 15 of follicular persistence (Fig. 2C, D, E, F and Fig. 3C). There were no differences ($P > 0.05$) in the power Doppler signals of blood flow between the follicular wall at proestrus in the PG and the follicular wall at proestrus in the CG (Figs. 2A, B and 3 C).

3.3. Blood flow in the ovarian artery

The blood flow velocity waveforms in a branch of the ovarian artery located in the ovarian pedicle was detected in all cows that were included in the study. The ED and TAMV measurements were low from the beginning of follicular persistence protocol (PG) and the values remained low at all stages of persistence when compared with the velocities in the CG at proestrus ($P < 0.05$) (Fig. 4B and C). The PS was of a similar pattern ($P < 0.021$) as the ED and TAMV, except for the PS at day 15 of follicular persistence (P15) where there were no differences in the CG at proestrus (Fig. 4A). Semi-quantitative evaluations of the vascular blood flow resistance using the Doppler indices (PI, RI and S/D) indicated there were no differences between the PG and CG ($P > 0.05$) (Fig. 4D–F). The indices were similar at all the stages of follicular persistence as compared to that of the CG at proestrus.

4. Discussion

In a previous study, persistent follicles were induced by long term administration of progesterone and these follicles had similar characteristics as those classified as follicular cysts. Many endocrine and morphological aspects as well as endocrine profiles, growth dynamics, and histological characteristics of persistent follicles were similar to those of spontaneous cysts (Díaz et al., 2015). In the present study, where there was evaluation of the hemodynamic changes that take place during a protocol of long term follicular persistence, there was no significant variation in the values when there was semi-quantitative evaluation of blood flow in the parenchyma of both the right and left ovaries between the PG and CG. In this regard, the presence of a regressing corpora lutea and/or dominant preovulatory or persistent follicle, as expected in each group, did not modify the total blood irrigation area. Results of some studies indicate dominant follicles and functional corpora lutea have a differential and greater amount of blood vascular network than other ovarian structures such as medium or small follicles and regressing corpora lutea (Acosta et al., 2005; Matsui and Miyamoto, 2009; Lansbergen, 2013). In the present study, however, there were no differences in the blood flow in the ovarian parenchyma, probably because the blood irrigation evaluations were conducted during proestrus and during follicular persistence, in the absence of a functional corpus luteum.

One of the most important findings of the present study is that the power Doppler signals of blood flow in the wall of persistent follicles from day 0 to day 15 of follicular persistence were less than in the CG, with the smallest irrigation area occurring on P15.

This decrease from P0 to P15 in the irrigation area in the wall of persistent follicles is a characteristic analogous to that reported previously in cows with spontaneous COD (Miyamoto et al., 2006; Rauch et al., 2008). Miyamoto et al. (2006) confirmed there was a greater blood flow in luteal compared with follicular cysts. These results are consistent with the findings by Rauch et al. (2008) where cows with follicles that had a wall thickness of < 3 mm and a progesterone concentration > 1.0 ng/ml, indicative that active luteal tissue, had a greater follicular blood flow than cows with follicles that did not contain luteal tissue. The growth of new blood vessels and establishment of a functional blood supply are important for development and function of the ovary (Fraser and Lunn, 2000; Berisha et al., 2010, 2013, 2016). Follicular growth and corpus luteum formation are achieved by repeated patterns of cellular proliferation, differentiation and transformation and a functional blood supply is needed for these developments to occur (Berisha et al., 2016). The small amount of blood irrigation area detected in the present study is consistent with the microscopic changes (apoptotic changes, loss of granulosa cell layers, no signs of luteinization, little evidence of a basement membrane, and reduced thickness of the granulosa and theca layers) previously observed in the walls of persistent follicles (Díaz et al., 2015). Furthermore, persistent follicles at the early stage of development (P0) have a sustained cell proliferation (expression of the Ki-67 protein was similar in P0 and control preovulatory follicles), with very little apoptosis and a greater cell survival (Belotti et al., 2017). From and subsequent to day 5 of follicular persistence in this previous study cell proliferation was less and the abundance of the apoptotic proteins cysteine aspartic proteases-3 (Caspase-3) and BCL-2 associated X protein (BAX) was greater than in preovulatory follicles (Belotti et al., 2017). There was a greater interleukin (IL)-6, IL-8 and tumor necrosis factor- α (TNF- α) protein expression at P0 in the previous study than that in preovulatory follicles. The IL-6 protein expression was greater in theca cells of persistent follicles on P10 as compared with preovulatory follicles from the control group. Additionally, IL-8 protein expression was greater in granulosa cells of persistent follicles from P0, P5 and P15 compared to that of preovulatory follicles of the control group (Stassi et al., 2017). The altered expression of angiogenic factors and cellular proliferation and apoptosis when there is development of persistent follicles could contribute to the vascular changes observed both in histological and ultrasonographic images (Díaz et al., 2015; Stassi et al., 2017, 2018). Interestingly, Isobe et al. (2005) reported that cystic follicles have a relatively greater vascular endothelial growth factor (VEGF) expression and an equally developed vasculature network in the theca interna, particularly in cystic follicles containing granulosa cells compared with healthy antral follicles. All this information suggests that the development and vitality of both persistent and cystic follicles are closely related to the angiogenic process.

The effectiveness of the hormonal treatment of COD depends on the presence of a functional luteinizing hormone receptor (LHCGR), and an increase in angiogenic factors, an increase in follicular blood flow, and a preservation of follicular cell integrity. As the persistence time progresses, the alterations in follicular blood flow together with changes in the development and functions of follicular cells, could partially explain the variability in the ovarian blood flow responses after hormonal treatment observed in animals with spontaneous COD (Rauch et al., 2008; Marelli et al., 2014). The evaluation of the intensity of blood flow with use of power Doppler could provide information about the viability of persistent or cystic follicles, and could contribute to a more precise diagnosis and therapeutic approaches in assessing these pathologies.

With the spectral Doppler (PW) examination, there was a lesser ED, TAMV and PS in the PG compared with the CG. This finding would indicate that persistent follicles induced by small doses of progesterone have lesser blood velocities in an arterial branch of the ovarian artery. Nevertheless, these changes were not accompanied by changes in the RI, PI, or S/D. The Doppler shift is affected by the insonation angle between the Doppler beam and the blood flow direction; therefore, the calculation of the blood flow velocity requires knowledge of the vessel orientation and a good immobilization of the patient during the exam, which is frequently difficult to achieve in large animals (Ginther, 2007; Viana et al., 2013). Furthermore, the Doppler indices are independent of the angle of flow relative to the face of the transducer and independent of the tortuosity of the ovarian arterial branches (Ginther, 2007; Ginther et al., 2016). Consistent with the findings of Ginther (2007) and Viana et al. (2013), it is postulated that the results observed for ED, TAMV and PS in the present study could have been affected by variations in the insonation angle during the exams, and that the Doppler indices allow for a more precise approach to evaluate ovarian perfusion in large animals.

5. Conclusions

Together with previous findings, the present results indicate that the irrigation of the walls of persistent follicles undergoes marked changes during the 15 days of follicular persistence, and that this could be a cause of the alterations that occur in the follicular development and function during early COD development. Furthermore, the use of power Doppler to evaluate the intensity of blood flow could be a technique that can be used to determine the viability of persistent or cystic follicles, and could contribute to a more precise diagnosis and therapeutic approach in determining and treating of these ovarian pathologies.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Acknowledgments

This study was supported by grants from the Argentine National Agency for the Promotion of Science and Technology (ANPCyT) (PICT-2016-3233; 2017-2821) and from the Universidad Nacional del Litoral (CAI + D 2016-50020150100067LI). PUD, NRS, BEM, HHO are Research Career Members and EMB, USN, CJML, LID are Fellows of the National Scientific Research Council (CONICET, Argentina). MLS is Postdoctoral Associate in the Department of Animal Science at Cornell University. We are grateful to the staff of

the Hospital de Salud Animal of the FCV UNL for the animal care and help with the generation of experimental animals and collection of images. We also thank the staff members of the Laboratorio de Biología Celular y Molecular Aplicada of the Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, for their technical support during the development of the experimental model. We thank F. Barberis and M. Houriet for providing the animals and for the assistance with animal care. We also thank Biogénesis Bagó, Zoovet and Over Laboratories for providing the drugs used in the protocols.

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