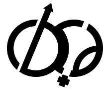

Three-dimensional modeling of color Doppler images: a new approach to study follicular vascularization in cattle

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Abstract

Ovarian blood supply is directly related to follicle developmental potential and to oocyte quality, and color Doppler ultrasonography might be a valuable tool to predict *in vitro* fertilization outcomes. In most studies in large domestic animals, however, the evaluation of follicle blood flow is qualitative (presence or absence of color signal) or dependent on the analysis of a single image. The objective of the present study was to first describe the use of a three-dimensional (3D) modeling of color Doppler images for a quantitative assessment of vascularization in bovine ovarian follicles. Follicular wave emergence was synchronized in Holstein and Gir heifers (n = 20), and follicular dynamics were assessed every 12 h using a color Doppler ultrasound device. The recorded cine-loop of the dominant follicle was decomposed into frames and medical image processing software was used to isolate the Doppler signal, generate the 3D model and calculate the volume of vascularization. In experiment 1, the model was validated by comparing the expected and calculated volumes and was used to predict possible variations in the results of the 2D approach. In experiment 2, vascularization was analyzed during follicular development. In both breeds, the volume of vascularization increased after follicle deviation and was positively correlated ($P < 0.05$) to follicular diameter (r = 0.65 and 0.54 for Holstein and Gir heifers, respectively). Spatial analysis of the three-dimensional model showed an uneven distribution of vascularization in the follicular wall, with a more intense blood flow being detected in the basal (nearest the ovarian hilus) and lateral regions of the dominant follicles. These results demonstrate the potential of this technique as a new tool for *in vivo* studies of ovarian physiology in large animals.

Keywords: bovine, blood flow, color Doppler, follicle development, follicular dynamics.

Introduction

Vascularization is directly related to ovarian follicle development. The growth of the capillary network in the theca layer is required for follicular antrum formation (Rodgers and Irving-Rodgers, 2010) and affects the composition and oxygen concentration of follicular fluid, influencing oocyte developmental potential (Sutton *et al*., 2003). It was suggested that follicular blood supply is also important for follicle selection and dominance, as well as for the ovulation process (Matsui and Miyamoto, 2009). In humans, follicular vascularization has been used as an evaluation parameter in assisted-reproduction programs (Chui *et al*., 1997; Borini *et al*., 2001; Lozano *et al*., 2007). In veterinary medicine, a relationship between follicular vascularization and *in vitro* fertilization outcomes and pregnancy rates was demonstrated in cattle (Siddiqui *et al*., 2009a, b).

Color Doppler ultrasonography is a valuable tool to evaluate vascularity and blood flow in the reproductive tract of farm animals. The assessment of the vascular system of small structures such as ovarian follicles, however, may be difficult to perform. The evaluation of follicular blood flow velocity during the systolic peak and the end of diastole, and also the assessment of mean blood flow velocity, was previously reported in preovulatory follicles (Acosta *et al*., 2003). Nevertheless, as it is not possible to accurately determine the insonation angle (Doppler angle) in capillaries, the results are subject to considerable variation. Angle-independent measures (Resistence and Pulsatility Indices) are alternatives used to evaluate blood perfusion of preovulatory follicles (Siddiqui *et al*., 2009a, b). Additionally, it is also not possible to precisely measure the diameter of the capillaries in the follicular wall, making calculation of volume blood flow impractical.

An alternative approach to measuring vascularization of the follicular wall is the use of subjective methods, such as visual scoring (Chui *et al*., 1997; Silva *et al*., 2006). Although previous studies have reported that both approaches (subjective and objective) were highly correlated in the evaluation of corpus luteum blood flow in mares (Ginther *et al*., 2007), criteria for subjective scoring of vascularization in small follicles may be more difficult to standardize and consequently, the results may be prone to bias. Due to these limitations, follicular vascularization has been frequently studied based only on the identification of the

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presence or absence of color signal in the follicular wall (Acosta *et al*., 2005; Pancarci *et al*., 2012), or quantitatively estimated by the ratio of color signal: area of the ovarian structure (Acosta *et al*., 2004a). The morphology of blood vessels, however, is not uniform throughout the follicular wall (Jiang *et al*., 2002) and, depending on the position of the transducer, these tubular structures can be visualized on the monitor as a strip or a small dot. Consequently, we can speculate that the area of vascularization can be both over- or underestimated when measured in bidimensional images.

Using new three-dimensional (3D) imaging technologies, ultrasound scans of structures can be reconstructed into a 3D image, spatially rotated and evaluated from all different perspectives (Singh *et al*., 2003). 3D technology has been used to study fetal development and diagnose fetal malformation (Werner *et al*., 2010; Kotoyori *et al*., 2012), and was also recently proposed to quantitatively evaluate follicular vascularization in human medicine (Vlaisavljevic *et al*., 2010). This new approach is particularly interesting in large animal theriogenology, where other image diagnostic tools such as magnetic resonance imaging or computed tomography are practically unavailable. The use of three-dimensional technology for ovarian vascularization studies in live cattle, however, has not been addressed yet. The aim of the present study was to evaluate the potential of a quantitative method comparing three-dimensional (3D) modeling software and 2D color Doppler ultrasonography for use in ovarian vascularization studies in cattle.

Materials and Methods

Experimental design and animals

All experimental procedures using animals were approved by the Ethics for Animal Use Committee of the Embrapa Dairy Cattle Research Center (Protocol CEUA-CNPGL nº: 02/2011). The use of 3D reconstruction algorithms to study follicular vascularization in cattle was evaluated in two experiments. In experiment 1, the use of a software-based procedure for 3D model reconstruction and volumetric measurements was validated. Additionally, the 3D model was used to simulate the potential variation of blood flow area measurement on 2D images. For this purpose, B-mode and color Doppler images from one Holstein cow were used. In the second experiment, vascularization detected by color Doppler ultrasonography in the wall of the dominant follicle was quantified using the 3D reconstruction procedure. In this experiment, Holstein (n = 10, average weight and age of 404.2 ± 71.5 kg and 18.3 ± 5.4 months, respectively) and Gir (n = 10, average weight and age of 358.9 ± 32.9 kg and 28.0 ± 4.8 months, respectively) heifers were used. All animals showed regular estrous cycles and no reproductive abnormalities were detected in previous gynecological exams. Animals were confined in free-stalls and fed corn silage and concentrate. Gir heifers were kept in an outdoor grazing system (*Brachiaria decumbens*) supplemented with corn silage and concentrate. Water, salt and mineral mixture were available *ad libitum*.

Follicular wave synchronization, B-mode and color Doppler ultrasonography

Ovarian follicular wave emergence was synchronized using estradiol benzoate (2.0 mg i.m., Sincrodiol, Ourofino Agronegocio, São Paulo, Brazil) and insertion of an intravaginal progesterone releasing device (1.0 g, Sincrogest, Ourofino Agronegocio, São Paulo, Brazil). The ovaries of the cow (experiment 1) and of the heifers (experiment 2) were examined by ultrasonography every 24 h using a duplex B-mode and color Doppler ultrasound machine (MyLab30 Vet Gold, Esaote, Genova, Italy) equipped with a 7.5 MHz trans-rectal transducer. The characterization of the emergence of the follicular waves and the selection of the dominant follicle were previously described (Viana *et al*., 2010). The ovaries were examined with B-mode every 24 h to detect the emergence of the new follicular wave (day 0), and thereafter B-mode and color Doppler ultrasonography were performed every 12 h to evaluate follicular vascularization until follicular dominance was clearly established (day 6). Before deviation, all growing follicles with a diameter ≥4 mm were evaluated and the dominant follicle was retrospectively identified based on follicular growth monitoring data. During each examination, follicle diameter was measured in a frozen B-mode image using the internal calipers of the machine, and then the color-flow mode (CFM, Doppler) was activated to visualize the presence of blood flow in the follicular wall. Color Doppler ultrasonography was performed with a pulse repetition frequency (PRF) of 0.7 KHz, so blood flowing with velocities greater than 0.05 m/s was observed as a colored area in the follicular wall. A gain of 70% and a low wall filter were used; these settings remained the same during all experimentation periods. With the CFM activated, the entire follicle was scanned (from one side to the other) and a 20 sec of video was recorded. To avoid spatial distortion of the 3D model and consequent miscalculation of the volume, a slow, continuous and unidirectional movement of the transducer was performed during the recording. All ultrasonography exams were done by the same technician (Arashiro, EKN). In experiment 2, the day and size (mm) of the dominant follicle at deviation were recorded and later used as physiological references for the interpretation of vascularization data.

Image processing for 3D modeling of follicles and validation of the reconstruction procedure

The recorded B-mode videos were decomposed into a sequence of frames and only images of the future dominant or dominant follicle were used to generate the

three-dimensional models with medical image processing software (Mimics 8.13, Materialise, Belgium). This software was developed for the segmentation of 3D images and engineering of anatomy models in human medicine (Werner *et al*., 2010). Using this software, the follicular antrum region was selected in each frame (Fig. 1A and 1B). Then, a 3D model was generated (Fig. 1C) and the follicular volume was calculated. To maintain the spatial resolution of the 3D model, the distance between frames was manually set in the software. This distance was obtained after dividing the follicular diameter by the number of frames. To validate the methodology, the volume calculated by the software was compared to the expected volume of a sphere ($4/3\pi r^3$, r = diameter/2 and π = 3.1416) with the same diameter of the follicle.

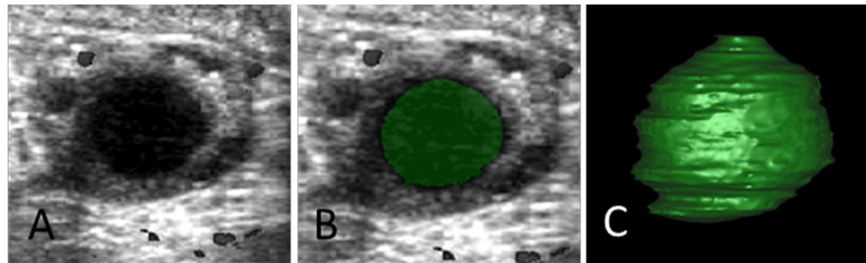

Figure 1. A: Frame showing the follicular antrum region (black). B: Follicular antrum region was selected using the 3D modeling software (Mimics 8.13; green). C: 3D model was generated and the follicular volume was calculated from selected frames.

Image processing for 3D modeling of follicular vascularization

The recorded color Doppler videos were decomposed into a sequence of frames as previously described and used to generate the three-dimensional models. The version of the 3D reconstruction software used did not recognize colored images, therefore before 3D modeling the area with blood flow signal in the follicular wall was marked white in each frame using the image software Adobe Photoshop 5.5 (Adobe Systems Incorporated, CA, USA). Doppler signal artifacts caused by the movement of the animal or its abdominal contents were not marked. The sequence of frames was then used to generate a three-dimensional model of the follicle and its respective blood vessels (Fig. 2A-D) and the volume of vascularization in the follicular wall was calculated.

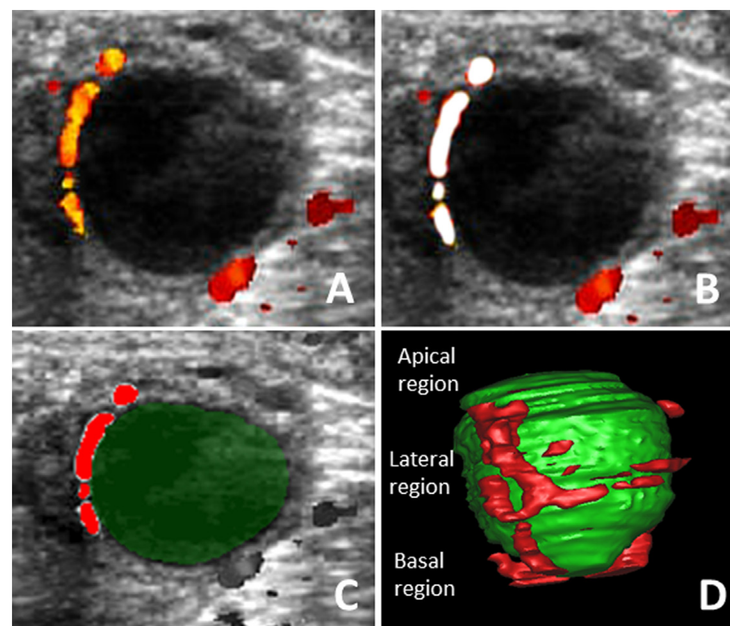

Figure 2. Three-dimensional model of a dominant follicle (13 mm in diameter) of a Holstein heifer. The frames were decomposed (A); blood flow area in the follicular wall was marked as white (B); follicular antrum and vascularization area colors were redefined (green and red, respectively, C); and the sequence of frames was used to generate a three-dimensional model of the follicle and its respective blood vessels (D).

Use of the 3D model to simulate 2D measurement results

A 3D model of follicular vascularization was used to simulate potential variation in the results of the evaluation of follicle vascularization based on blood flow area measurement in 2D images. The 3D model was rotated on its vertical axis and cross-section slices were made at different angles, but always at its maximum diameter (Fig. 3) using commercial NURBS-based 3-D modeling software (Rhinoceros 5, McNeel North America, Seattle, WA, USA). The area of vascularization was measured in each cross-section. Additionally, in the cross-sections with the smallest and largest vascularization areas (30/210° and 150/330°, respectively) two additional slices were made at 50% of the maximum diameter in each direction (Fig. 4), and the area of vascularization was also measured in these cross-sections.

Three-dimensional modeling (experiment 1 and 2) was performed at the Laboratory of Three-dimensional Models at the National Institute of Technology, Rio de Janeiro, Brazil. For statistical analyses, only data related to dominant follicles were used.

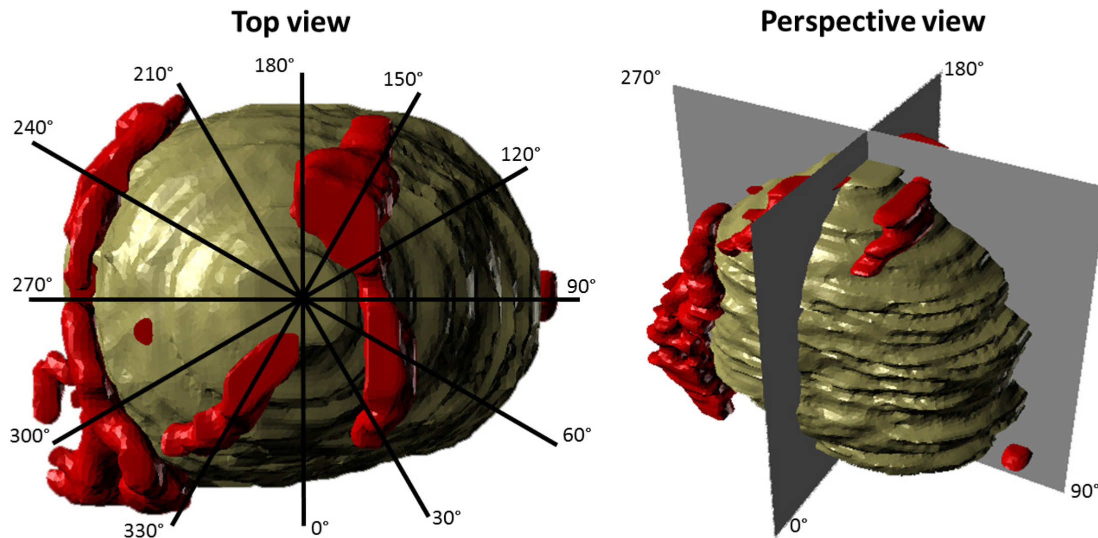

Figure 3. Top and perspective views of a three-dimensional model of a dominant follicle showing the angle slices used to generated the cross-sections in which the area of vascularization was measured.

Figure 4. Top view of a three-dimensional model of a dominant follicle showing the slices performed at 50% of maximum diameter in either direction in relation to the cross-sections with the smallest or largest vascularization area (30-210° and 150-330°, respectively).

Statistical analyses

In validation methodology (experiment 1), variation between the volume calculated by the software (CV) and the expected volume (EV) was calculated by the formula: [(CV/EV) -1]*100. Data for follicular diameter and volume of vascularization were evaluated for normality and homoscedasticity using Lilliefors and Cochran and Bartlett tests and were normally distributed. The volume of vascularization during follicular growth was analyzed using the SAS GLM procedure with the main effect of breed (Gir or Holstein), repeated statement for hours relative to dominant follicle deviation, and the breed by hour interaction. Association between follicular diameter and volume of vascularization was determined using Pearson's correlation method. Results are presented as mean ± SEM. Statistical significance was declared at $P < 0.05$.

Results

In experiment 1, the difference between the volume of the dominant follicle (calculated by the 3D modeling software) and the expected volume (calculated by the formula $4/3\pi r^3$) ranged from 0.3 to 6.1% (Table 1). The values of vascularization area, as measured in different bidimensional images generated from the 3D model of the same follicle, ranged from 0.22 cm^2 to 12.36 cm^2 (slices 30-210° and 150-330°, respectively). The variation in the value of follicle blood flow area according to the angle of the cross-section is shown (Fig. 5). When the slices were performed at 50% of the maximum diameter in each direction, the area of vascularization was over or underestimated when compared to the area calculated at maximum follicular diameter in cross-sections 30-210° or 150-330°, respectively (Table 2).

Table 1. Expected (EV) and calculated (CV) volumes of ovarian follicles of different diameters.

Day	Follicular diameter (mm)	EV (mm^3)	CV (mm^3)	Variation (%)
D0	5.0	65.45	65.47	0.29
D1	7.4	212.18	220.44	3.89
D2	9.4	434.89	408.27	6.12
D3	10.9	678.08	649.63	4.20
D4	12.4	998.31	941.26	5.71
D5	13.6	1317.09	1263.21	4.09

EV, expected volume determined by the formula ($4/3\pi r^3$, r = diameter/2 and π = 3,1416). CV, calculated volume by the 3D modeling software (Mimics 8.13)

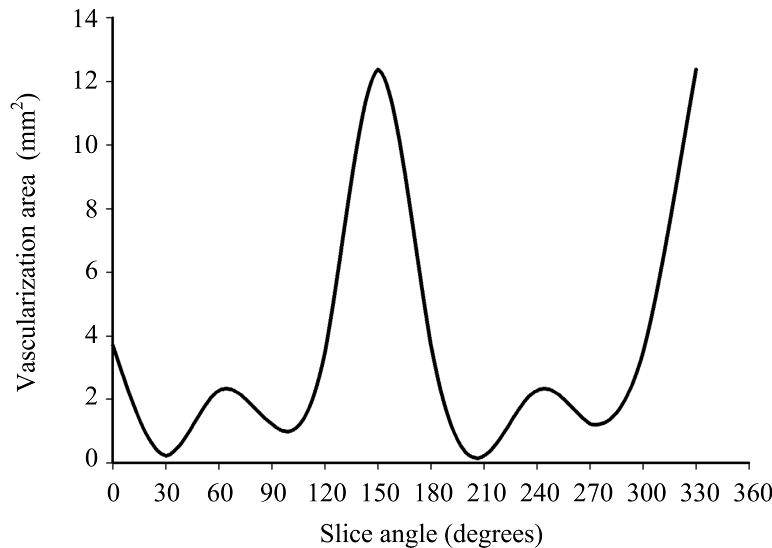

Figure 5. Variation of the values of vascularization area in 2D images generated from a 3D model of the same follicle at different angles.

Table 2. Vascularization area in cross-sections obtained at 50% of maximum diameter in either direction (left and right) of the cross-sections in which the smallest and the largest vascularization area were observed (30-210° and 150-330°, respectively).

	Vascularization area (cm^2)		
Slice	Maximum diameter	50% maximum diameter (left)	50% maximum diameter (right)
30-210°	0.23	2.61	4.48
150-330°	12.36	2.35	2.20

In both breeds, follicular deviation occurred 1.9 ± 0.2 days after follicular wave emergence. As expected, the diameter of the dominant follicle at deviation was larger in Holstein than in Gir heifers (8.7 ± 0.1 *vs*. 6.9 ± 0.5 mm, respectively; $P < 0.05$). Color Doppler signals were first observed when dominant follicle reached 4.7 ± 0.2 and 5.6 ± 0.5 mm in diameter in Holstein and Gir heifers, respectively. There was an effect of hour from deviation, but no breed by hour interaction. Volumes of vascularization at -12 to 48 h from deviation ranged from 25.6 ± 4.8 to 41.8 ± 5.3 mm^3 (27.5 ± 4.1 mm^3 at deviation) in Holstein heifers; and from 12.7 ± 7.3 to 32.2 ± 13.1 mm^3 (18.1 ± 7.4 mm^3 at deviation) in Gir heifers and increased ($P < 0.05$) after follicle deviation in both breeds (Fig. 6). A significant positive correlation between follicular diameter and volume of vascularization was observed in both breeds (r = 0.65 and 0.54 for Holstein and Gir animals, respectively; $P < 0.05$).

In the generated 3D models, the distribution of blood vessels along the follicular wall could be visualized and described. Blood flow was detected by color Doppler ultrasonography in the basal and lateral but not in the apical regions of the follicles (Fig. 2D), showing evidence of an uneven distribution of vascularization in the follicular wall.

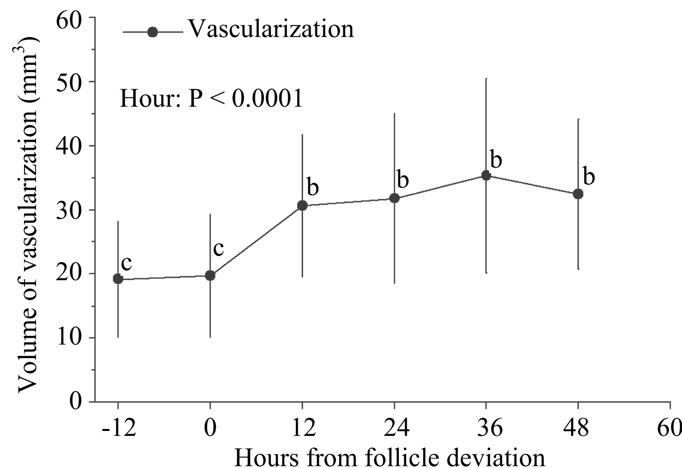

Figure 6. Volume of vascularization in dominant follicles relative to hour of deviation, calculated by three-dimensional modeling of color Doppler images.

Discussion

The present study used color Doppler ultrasonography images of dominant follicles to construct three-dimensional models for quantitative assessment of follicular vascularization using volumetric measurement. To our knowledge, this is the first study to describe the use of this novel approach to study follicular vascularization in cattle. During a conventional B-mode ultrasound exam, the evaluation of organs or tissues is performed by continuous visualization of bi-dimensional images (2D) and mental reconstruction of the third-dimension by the technician (Singh *et al*., 2003). In the present study, the association of color Doppler ultrasonography and a 3D reconstruction algorithm allowed the visualization, in a single 3D image, of all colored areas representing blood flow (greater than 0.05 m/s) in the follicular wall. Moreover, the analysis of the 3D image demonstrates the potential to be used in further studies regarding the characterization of the vascular distribution and architecture of the follicular wall.

The software Mimics® had already been used to generate 3D models of human fetuses from images obtained by 3D ultrasonography, magnetic resonance imaging and computed tomography (Werner *et al*., 2010). The results in Table 1 demonstrate that it was also possible to generate 3D models from images obtained by 2D ultrasonography with a reasonable volumetric accuracy and spatial distribution. Volumetric measurements require a 3D image without distortions in order to obtain reliable values. During the video recording, the scan was performed with a slow, continuous and unidirectional movement; otherwise, the spatial resolution of the follicle and its related vascularization would be distorted, reducing the accuracy of the volume calculated by the software. Consequently, the methodology used in the present study required careful animal restraint and diligent ovary scanning. Distortions are less likely to occur when volumetric transducers are used, but very few ultrasound devices currently used have transducers designed for 3D/4D scanning of the reproductive tract of large animals.

Using the methodology proposed in the present study, it was observed that the volume of vascularization in the follicular wall progressively increased throughout dominant follicle development. This increase was expected since there is temporally-dependent angiogenic activity during follicular growth

in cattle (Jiang *et al*., 2003), probably driven by the follicle's increasing metabolic requirements. Follicular blood supply is positively correlated with steroidogenic activity (Grazul-Bliska *et al*., 2007) and follicle vascularization, consequently, directly influences the selection of the dominant follicle (Acosta, 2007). To evaluate the coherence of the expected changes in follicle vascularity and the outputs of the 3D modeling, *Bos taurus* and *Bos indicus* dairy breeds (Holstein and Gir, respectively), in which follicle deviation occurs at different diameters (Kulick *et al*., 2001; Viana *et al*., 2010), were used as models. Follicular diameter and volume of vascularization were positively correlated in both Holstein and Gir heifers, and in both breeds, vascularization increased after the expected size at deviation (8.7 ± 0.1 and 6.9 ± 0.5 mm for Holstein and Gir, respectively) in a fashion similar to what occurs with estradiol production (Glister *et al*., 2006), indicating the potential use of vascularization measurement to evaluate follicular function.

The study of follicular vascularization in large animals using color Doppler ultrasonography was previously performed (Acosta *et al*., 2003). However, quantitative evaluation of color Doppler signals remains a challenge. The progressive increase in follicular vascularization observed in the present study using volumetric measurement corroborates with previous results obtained using colored area measurement in 2D images (Pancarci *et al*., 2011). Follicular vascularization was quantified in one or more 2D ultrasonography images measuring the area of color Doppler signals in the follicular wall (Acosta, 2004b; Gastal *et al*., 2007; Rauch *et al*., 2008; Pancarci *et al*., 2011). However, it is known that the vascular network of the ovarian follicle is different among follicular regions (Jiang *et al*., 2002, 2003); therefore, quantification of follicular vascularization in a single point may not be accurately representative, as demonstrated in the present study (Fig. 5 and Table 2). In this regard, to use 2D images to evaluate follicular vascularization, the position of the ovary during the ultrasonography exam must be taken into account. Furthermore, temporal evaluations may be difficult, since performing the examination at the same point every time may not be possible. Qualitative (presence or absence of color Doppler signals) and subjective (score 1-4) methodologies have also been used (Acosta *et al*., 2005; Silva *et al*., 2006); however, data interpretation is limited and the reproducibility of these methods is difficult. The methodology proposed in the present study was a new approach to study follicular vascularization that allowed the quantification of the color Doppler signals in the entire follicular wall, eliminating possible biases. Moreover, we have first established reference values for follicular vascularization volume during the peri-deviation period in *Bos taurus* and *Bos indicus* breeds. However, evaluation of low levels of blood flow in peri-deviation follicles demands an ultrasound scanner with high sensitivity which is affected by probe frequency, PRF, color-flow gain and wall filters.

With this new approach, it was also possible to visualize the distribution of blood vessels along the follicular wall. Blood flow was detected in the basal and lateral regions of the follicles, demonstrating the uneven organization of the vascular architecture in the follicle wall. The presence of large blood vessels (arteries, venules) in these regions (Jiang *et al*., 2002, 2003) resulted in a flow velocity detectable by color Doppler ultrasound. Similar findings were previously reported for preovulatory follicles in cattle (Acosta *et al*., 2003) and humans (Brännström *et al*., 1998), despite the different approaches used to evaluate the Doppler images. In the current study, however, the 3D reconstruction and the possibility of spatial rotation clearly demonstrated that the irregular distribution of this vascular mesh was not an artifact created in the 2D images, but it was in fact the real architecture of the follicular vessels. Therefore, this methodology offers an alternative approach for experimental studies of ovarian follicular and corpus luteum vascularization in large species, by allowing vascular volumetric measures to be performed using standard ultrasound/color Doppler devices not equipped with a 3D/4D transducer or licenses.

In conclusion, the results observed in the present study demonstrate the potential of the 3D modeling of color Doppler images for quantitative measurement of follicular vascularization and for the assessment of the vascular architecture in ovarian follicles, providing a new tool for *in vivo* studies of ovarian physiology in cattle and other domestic species.

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