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Relationships between follicle and corpus luteum size and vascularization with ovulation, progesterone production, and pregnancy in Nellore beef cattle

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ABSTRACT - The objective of this study was to evaluate the relationships between preovulatory follicle (POF) and corpus luteum (CL) diameters, and POF and CL vascular perfusion with progesterone production, ovulation, and pregnancy in Nellore cows subjected to timed artificial insemination (TAI). Nellore cows (n = 201) were subjected to ovulation synchronization and later to ultrasound evaluation of POF and CL at the time of insemination (D0) and seven days later (D7), respectively. Females were divided into three categories according to the POF diameter assessed at the time of insemination: small (SF), medium (MF), and large (LF) follicles. The LF group had a greater number and intensity of pixels in the POF ultrasound exam compared with the SF group. The CL flow intensity and progesterone concentration were also higher in the LF group. The SF group showed lower flow intensity and lower ovulation rate compared with the others. When non-pregnant females were compared to pregnant ones, no difference was observed in any of the analyzed variables. The results show for the first time in Nellore cattle the relationship between the size of ovarian structures and blood flow (quantity and intensity) as well as the ability of the CL to produce progesterone. The intensity of the POF pixels proved to be relevant, demonstrating correlations with the size and flow of the CL, which were not found when evaluating only the number of pixels, thus revealing the importance of evaluating complementary characteristics of the flow.

Keywords: cattle, color Doppler, ovary, preovulatory follicle, vascular perfusion

1. Introduction

The development of a healthy preovulatory follicle (POF) containing a competent oocyte is critical to the establishment of pregnancy. The POF size in cattle is one of the most important factors influencing reproductive success. The relationship between POF size and fertility in cattle has been the focus of many studies in recent years (Colazo et al., 2015; Mokhtari et al., 2016; Tarso et al., 2016).

In the search for better results, Doppler ultrasonography emerges as an important tool, and is a minimally invasive technique that supports reproductive management through the evaluation of vascular perfusion in the reproductive tract of cows during various stages of the estrous cycle, pregnancy, and puerperium. This assessment provides important additional information on the physiological and pathological processes in the uterus and ovaries (Herzog and Bollwein, 2007). Cyclic hemodynamic changes occur in the ovarian tissue of cows during late follicular growth, ovulation, and corpus luteum (CL) development (Acosta et al., 2003). The blood flow of these structures is directly related to their functionality (Lüttgenau and Bollwein, 2014). According to Siddiqui et al. (2009), animals with an intense flow in the follicular wall are also more likely to become pregnant after insemination.

One of the main points to understand the role of vasoactive substances in ovarian physiology is to determine the change in local blood flow in follicles and corpora lutea at specific stages of the estrous cycle. Vasoactive substances also play an important role in modulating the secretion of steroid hormones and prostaglandins (Acosta et al., 2000).

Assuming that blood flow changes in FOP and CL of different sizes could enable us to evaluate the functionality of these structures, and that such assessments have not yet been studied in Nellore cows, the present study tested the following hypotheses: larger POF form larger corpora lutea; larger follicles and corpora lutea present greater intensity of blood flow; POF with a greater quantity and intensity of blood flow when ovulated give rise to corpora lutea with greater quantity and intensity of blood flow and produce more progesterone; and females with greater amount and intensity of luteal blood flow have a higher pregnancy rate when subjected to timed artificial insemination (TAI). Thus, the present study aimed to evaluate the relationship between the diameter of the POF and the CL and vascular perfusion with progesterone production, ovulation, and pregnancy in Nellore cows subjected to TAI.

2. Material and Methods

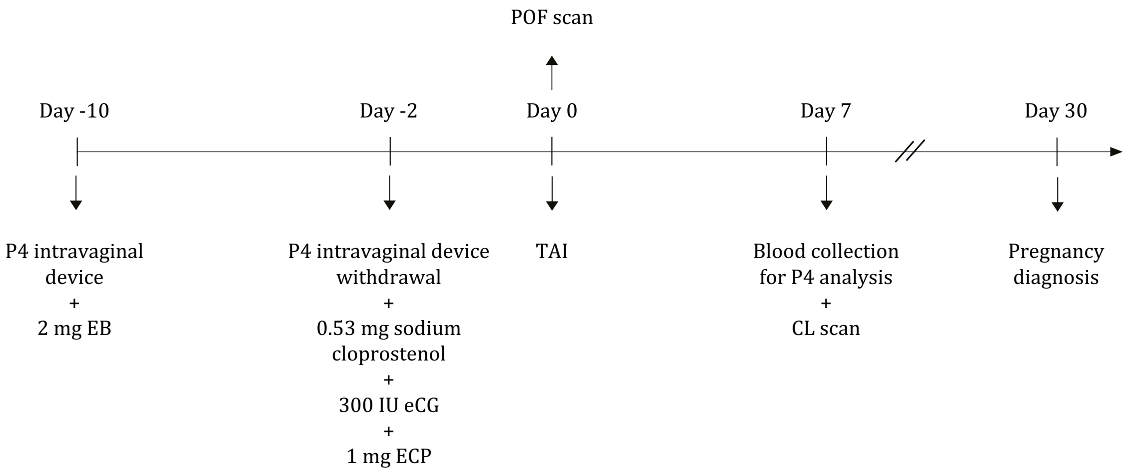
2.1. General experimental considerations

This study was approved by the Animal Ethics Committee (process no. 23083.008085/2018-34) and carried out on a commercial beef cattle farm located in the city of Paraibuna, São Paulo, southeastern Brazil (latitude: 23°23'10" S, longitude: 45°39'44" W). Two hundred and one pluriparous, non-pregnant, cycling and noncycling, lactating Nellore females, with average body condition score (BCS) of 3.0 (range of 2.0-4.0), were subjected to ovulation synchronization and TAI. The cows registered in the study ranged from 40 to 80 days postpartum. The animals were raised in an extensive environment with *Brachiaria brizantha* pasture, water, and mineral salt *ad libitum.* All animals used in the experiment were kept in a single group on the farm. At the time of procedures inside the working facility, they were subdivided into small lots (of approximately 30 animals each) to facilitate handling.

2.2. Ovulation synchronization and TAI

At the beginning of the ovulation synchronization protocol, the percentage of cycling cows (presence of corpus luteum) in the groups of small, medium, and large follicles were, respectively, 28.6, 38.1, and 42.9%. Females were synchronized using a hormonal protocol based on Guerson et al. (2021), in which, on a random day of the estrous cycle (start of treatment; D-10), a new 1 g progesterone intravaginal device was inserted, along with 2.0 mg of estradiol benzoate administered intramuscularly. The device was maintained in place for eight days and, when it was removed (D-2), 0.53 mg of sodium cloprostenol + 300 IU of equine chorionic gonadotropin + 1.0 mg of estradiol cypionate were administered intramuscularly. Timed artificial insemination was performed 48 h after the device was removed, and this day was called D0 (Figure 1).

At the end of the hormonal treatment, ovulation rate was estimated by dividing the number of animals in which a CL was observed in an ovary following examination with transrectal ultrasonography, seven days after artificial insemination (AI), by the total number of inseminated animals.



POF - preovulatory follicle; P4 - progesterone; IU - international units; EB - estradiol benzoate; eCG - equine chorionic gonadotrophin; ECP - estradiol cypionate; TAI - timed artificial insemination; CL - corpus luteum.

Figure 1 - Schematic representation of the experimental procedures.

2.3. Ultrasound evaluation and pregnancy diagnosis

Transrectal ultrasound evaluation of the ovaries was performed with a Mindray Doppler device, model Z5VET, equipped with a 75L50EAV linear transducer and 5.0/7.5 Mhz wave emission, standardizing a total gain of 70%. The images of the POF and CL were performed in B-mode to determine the positions and measurement of these structures. The diameter was calculated by the average of the two largest transversal measurements perpendicular to each other. The ultrasound evaluation of the POF was performed before insemination (D0) and that of the CL, seven days after TAI (D7) (Figure 1). Based on the measurement of the all POF diameter, the females were divided into three groups as described by Pugliesi et al. (2016): the SF group of females have a small follicle, with a diameter < 1.1 cm (n = 35); the MF group of females have a medium follicle, with diameter between 1.1 and 1.4 cm (n = 84); and the LF group of females have a large follicle, with a diameter ≥ 1.4 cm (n = 70).

The images in the color Doppler mode were recorded in AVI video format and stored on a magnetic disk for further analysis. The videos were analyzed by selecting three still images, to obtain an average of the variables, which showed the POF or CL with its largest diameter and the largest blood flow. Subsequently, the captured images were edited and subjected to Image J software analysis to quantify the number of colored pixels present (Ginther, 2014) (Figure 2). All ultrasound evaluations in this study were performed by a single operator.

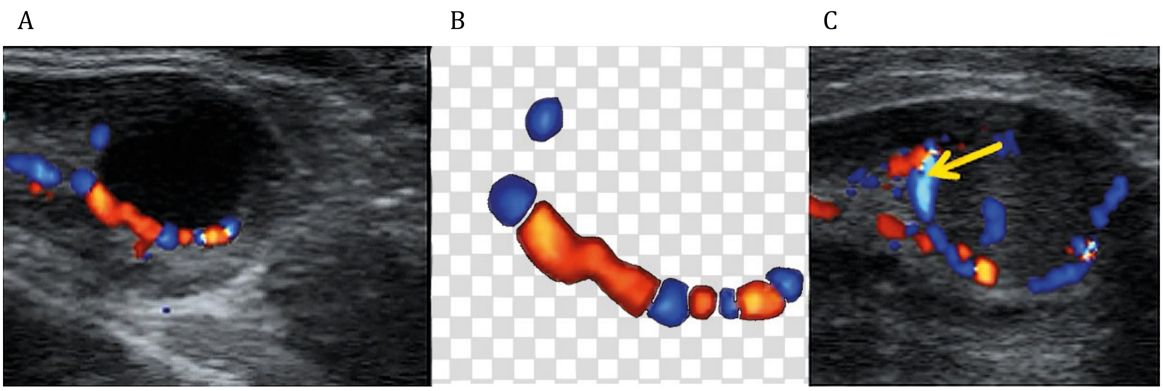


Figure 2 - Preovulatory follicle with the presence of blood flow in the color Doppler mode (A); edited image showing only the areas of color flow, for subsequent pixel counting (B); and corpus luteum seven days after insemination with the presence of flow (C).

During the pixel count, the flow intensity was quantified through an equation, using the number of pixels found for each color tone multiplied by the numerical code that represents each of these tones present in the image (Guerson et al., 2021). The sum of these products gave the flow intensity, which is directly linked to the blood flow velocity.

Pregnancy diagnosis was performed 30 days after insemination using transrectal ultrasonography (linear transducer; 7.5 MHz). Confirmation of pregnancy was based on visualization of the embryonic vesicle with the presence of a viable embryo (presence of a heartbeat).

2.4. Progesterone assay

On the seventh day after artificial insemination, blood samples were collected with an evacuated tube without anticoagulant from the coccygeal vein or artery for subsequent measurement of the concentration of progesterone in serum. The samples were centrifuged for 15 min at 480 *g*, and the serum was kept refrigerated at −20 °C until further analysis. Serum P4 values were evaluated by the solid-phase radioimmunoassay technique, using commercial kits (ImmuChem, MP Biomedicals, Santa Ana, California, USA). The samples were analyzed in a unique assay with a sensitivity and intra-assay coefficient of variation of 0.05 ng/mL and 11%, respectively. All data were within the minimum and maximum points of the curve.

2.5. Statistical analyses

Statistical analysis was performed using the software R., v. 3.6.1. A general linear model was implemented (glm function of the stats package) with different error structures (Gaussian or gamma) and link functions (identity). The error structure was evaluated using the descdist function from the fitdistrplus package. Data were first evaluated for normality using the Shapiro-Wilk test. Data were subjected to ANOVA test, and multiple comparisons between treatments were conducted using Tukey contrasts through the emmeans function of the emmeans package. The variable progesterone concentration was not normally distributed; therefore, the groups were compared using the non-parametric Kruskal-Wallis test. The significance level considered to indicate an effect of the categorical variables was $P \leq 0.05$ and $0.05 < P \leq 0.10$ was considered a trend. The variables were related using Pearson's correlation. Data were expressed as mean and SEM (standard error of the mean) unless otherwise stated.

The statistical model used can be described by the following equation:

$$Y_{ij} = \mu + T_j + e_{ij},$$

in which Y_{ij} = observed value of the response variable in the experimental unit, μ = general mean of the experiment, T_j = treatment effect, and e_{ij} = is the error that follows the gaussian or gamma family.

3. Results

Significant differences were found in the mean diameter of the POF and the CL between the three groups (SF, MF, and LF; Table 1). Animals within the LF group had significantly greater mean diameters of the POF and CL following ovulation and greater circulating concentrations of P4 compared with animals in the SF and MF groups.

Positive correlations were observed, ranging from weak to moderate, for the diameter of the POF and the CL and all other variables (Table 2). The number of follicle pixels showed a weak positive correlation with its diameter (r = 0.16) and, as expected, moderate with intensity (R = 0.48). The follicle size showed a moderate positive correlation (r = 0.49) with the CL diameter. In relation to the number of pixels and the intensity of the POF as seen on the color Doppler exam, the mean vascular perfusion of the SF group was significantly lower than that of the LF group. This difference in the number of pixels and the intensity between small and large follicles were also observed when these variables were

Table 1 - Means and standard deviations of follicle (FL) and corpus luteum (CL) variables of Nellore cows divided into three groups according to potential follicular diameters[1]

Parameter	SF group (n = 35)	MF group (n = 84)	LF group (n = 70)
FL diameter (cm)	0.96±0.09a	1.26±0.01b	1.58±0.11c
Number of colored pixels - FL	329±49a	448±31.8ab	556±35.1b
Pixel intensity[2] - FL	36872±24711a	50368±31028b	61670±31811b
CL D7 diameter (cm)	1.78±0.05a	1.92±0.03b	2.14±0.03c
Number of colored pixels - CL D7	1493±130a	2233±113b	2348±134b
Pixel intensity[2] - CL D7	167823±15128a	248845±13114a	265544±15670b
Concentration P4 (ng/mL)	1.18±0.19a	1.57±0.12a	2.17±0.16b
Ovulation rate (%)	77.1a	95.2b	94.3b
Pregnancy rate (%)	43.5a	56.4a	50a

D7 - day 7 after ovulation; P4 - progesterone.
[1] SF - small follicles: diameter < 11 mm; MF - medium follicles: diameter between 11 and 14 mm; LF - large follicles: diameter ≥ 14 mm.
[2] Intensity is a numerical value obtained from the numerical code of each color multiplied by the number of pixels of that color, with no specific unit of measurement.
Different letters indicate statistical difference (P<0.05).

Table 2 - Correlation between variables related to follicle (FL) and corpus luteum (CL) and concentration of progesterone in Nellore cows subjected to timed artificial insemination

	FL Ø (cm)	FL intens.	FL pixel	CL Ø (cm)	CL intens.	CL pixel	[P4]
FL Ø (cm)		r = 0.32**	r = 0.16**	r = 0.49**	r = 0.25**	r = 0.26**	r = 0.23**
FL intens.			r = 0.48**	r = 0.26**	r = 0.27**	r = 0.26**	r = 0.08ns
FL pixel					r = 0.10ns	r = 0.09ns	r = 0.08ns
CL Ø (cm)					r = 0.37**	r = 0.37**	r = 0.32**
CL intens.						r = 0.97**	r = 0.11ns
CL D7 pixel							r = 0.11ns
[P4]							

FL Ø - follicle diameter; FL intens. - follicle flow intensity; FL pixel - amount of follicle pixels; CL Ø - corpus luteum diameter on day seven after insemination; CL intens. - corpus luteum flow intensity on day seven after insemination; CL pixel - amount of corpus luteum pixels on day seven after insemination; [P4] - serum progesterone concentration on day seven after insemination.
ns - not statistically significant; ** - statistically significant at P<0.01; * - statistically significant at P<0.05.

compared for the CL. There was no difference in the number of pixels and flow intensity between MF and LF. The numbers of CL pixels were also similar for the MF and LF groups, although the LF showed a higher flow intensity.

Cows in the SF group had a lower ovulation rate compared with animals in the other groups. Despite this, no significant difference was observed between the pregnancy rates of the three groups. We compared the same variables in Table 1 between non-pregnant and pregnant females (Table 3) and observed no significant difference for any of the parameters evaluated.

4. Discussion

The present study is the first in female *Bos indicus* (Nellore) to indicate that the size and blood flow of the preovulatory follicle close to ovulation positively affect the size and vascularization of the subsequent CL and its ability to produce P4, seven days after ovulation.

Positive correlations were observed, ranging from weak to moderate, for the POF and CL diameter and all other variables. According to Vasconcelos et al. (2013), the POF diameter is involved in a balance

Table 3 - Means and standard deviations of variables related to the follicle (FL) and corpus luteum (CL) and the concentration of progesterone between non-pregnant and pregnant Nellore cows

Parameter	Non-pregnant (n = 92 / 48.7%)	Pregnant (n = 97 / 51.3%)	P-value
FL diameter (cm)	1.28±0.28	1.33±0.24	0.33
Number of colored pixels - FL	456±277	502±275	0.36
Pixel intensity[1] - FL	51184±32661	56081±32158	0.47
CL D7 diameter (cm)	1.98±0.30	1.98±0.28	0.98
Number of colored pixels - CL D7	2354±938	2488±1076	0.62
Pixel intensity - CL D7	265326±106099	280227±130692	0.69
[P4] (ng/mL)	1.86±1.13	1.93±1.15	0.80

D7 - day 7 after ovulation; [P4] - serum progesterone concentration on day seven after insemination.
[1] Intensity is a numerical value obtained from the numerical code of each color multiplied by the number of pixels of that color, with no specific unit of measurement.

between oocyte quality, an adequate level of circulating estrogen close to insemination, and adequate circulation of progesterone after AI.

Regarding the positive correlation between follicle size and CL diameter (Lonergan et al., 2013; Pinaffi et al., 2015; Pugliesi et al., 2019), larger follicles are expected to give rise to larger corpora lutea, which have a greater capacity to produce progesterone, and they are associated with higher pregnancy rates after TAI. Additionally, the differences found between the SF, MF, and LF groups for follicle diameter were repeated for the same CL variables. Tarso et al. (2016) stated that the POF dimensions can be used to estimate the CL dimensions. At day 7, while the CL is still growing, the mean of CL diameter did not differ significantly between pregnant and non-pregnant females, and the pregnancy rate did not differ between the SF, MF, and LF groups, although Perry et al. (2007) associated larger follicle sizes with higher pregnancy rates.

The ovulation rate was less in the SF group compared with the groups with larger follicle sizes, which supports the findings of others that the presence of developing follicles with larger diameters is associated with a greater probability of ovulation (Butler et al., 2011).

Circulating concentrations of progesterone were significantly higher in the LF group (follicles > 14 mm) compared with the other groups. This was in accordance with Vasconcelos et al. (2013), who also observed higher concentrations of progesterone on day 7 in cows that ovulated larger follicles (> 17 mm), and according to them, the optimal follicle size is related to an adequate post-AI P4 circulation. Although positive, weak correlations were found in the present study between follicle and CL dimensions and the amount of progesterone on day 7, in contradiction to the findings of Tarso et al. (2017), who found high correlations between the POF and CL dimensions and the blood flow of both structures, as well as with the plasma concentration of progesterone. A possible explanation for these differences is the different animal breeds in the two studies (Nellore *versus* Aberdeen Angus) since *Bos taurus* cows have lower POF and CL than *Bos indicus* (Sartori et al., 2010). Furthermore, another factor that may explain this difference is that, in the present study, blood flow and progesterone concentrations were evaluated seven days after insemination and not after ovulation as in the experiment by Tarso et al. (2017). In addition, seven days after insemination, progesterone levels are still rising.

Although the LF group had a higher progesterone concentration in relation to the other groups, the pregnancy rate did not differ between any of them. This demonstrated that the progesterone concentrations were satisfactory to maintain pregnancy in all groups and that larger follicles do not always result in higher pregnancy rates. In some cases, larger follicle size may be associated with persistence of dominance (Colazo et al., 2015).

Despite the difference observed in the progesterone concentration in the LF group in relation to the others, in the present study there was no difference (P = 0.80) in the progesterone concentrations between non-pregnant and pregnant females. However, it is important to highlight that the experimental design (blood collection seven days after AI and not after ovulation) may not have been adequate to determine such a difference. Even so, this finding is in agreement with Pugliesi et al. (2019), who also found no difference in serum progesterone on day 7 in bovine embryo recipients that became pregnant or not, and stated that luteal blood perfusion is a better indicator of luteal activity and probability of becoming pregnant in relation to P4 concentration.

Another finding in common with the present study is that the size of the CL was also similar between pregnant and non-pregnant cows. This finding could possibly be explained by the variations in size, blood flow, and progesterone concentration during the luteal phase that have been recorded. Herzog et al. (2010) divided the luteal period into three phases: luteal growth phase, luteal static phase, and luteal regression phase. During the luteal growth phase, progesterone concentration, luteal size, and luteal blood flow increased almost identically. However, differences were observed in the static phase, with only a moderate increase in luteal size, while blood flow and progesterone concentrations increased almost equally. It could be that differences between pregnant and non-pregnant animals may have only become apparent during the static or regressing phases and could explain why differences in luteal size, blood flow, and circulating concentrations of progesterone were not recorded in this study when measurements were taken on day 7 (which did not reflect day 7 after ovulation), which was during the luteal growth phase. Such relationships between luteal blood flow and progesterone concentrations make vascular perfusion the main indicator of changes in progesterone concentration throughout the cow cycle (Herzog et al., 2010; Lüttgenau et al., 2011; Thijssen et al., 2011). Andrade et al. (2019) found that the CL diameter presented the same efficacy as vascular perfusion for the pregnancy diagnosis in Nellore heifers; however, these analyzes were performed only on day 21 after AI. Lüttgenau and Bollwein (2014) stated that there are important relationships among blood flow, hormone production capacity, and morphology associated with improved fertility.

In the present study, the SF group had a lower number of pixels compared with the other groups, attesting that lower blood perfusion is associated with smaller diameters, as previously observed by Pinaffi et al. (2015) and Tarso et al. (2016). Thus, the lower the vascular perfusion, consequently there is less supply of gonadotropins, which are biochemical substances and hormonal factors necessary for follicular development (Acosta and Myamoto, 2004).

Although Siddiqui et al. (2009) associated increased follicular vascularization with a greater chance of establishing pregnancy, the conception rate in the present study did not differ between groups (P = 0.36), nor did the number of pixels in the follicle wall differ between pregnant and non-pregnant females. Another important fact that may have influenced the present result is that some cows, even when not pregnant, could have a high pixel count, since according to Pinaffi et al. (2015), and in a contradictory way, CL from cows with blood flow greater than 70% in the subjective analysis showed a decrease in fertility.

Although the number of colored pixels, which represent the blood flow of the CL, was higher in animals with medium (MF) and large (LF) follicles in relation to those with small follicles (SF), this characteristic did not differ (P = 0.62) between the groups of non-pregnant and pregnant females in the present study. Therefore, according to Pugliesi et al. (2019), the blood perfusion of the CL is the main factor to be evaluated in embryo recipients, directly affecting the probability of pregnancy. Luteal blood flow shows temporal variations during the estrous cycle, and this may have contributed to the inexistence of differences between them, since the evaluations were performed on day 7 after estrus and, according to Herzog et al. (2011), until day 15 after estrus, blood flow was also similar between pregnant and non-pregnant cows.

In the present study, no differences were found in the number of pixels between the SF and MF groups; therefore, the flow intensity differed between these two follicle categories. The SF group with less intensity and smaller follicular diameters reinforces the idea of a positive correlation between follicular size and its vascular perfusion, as demonstrated by Tarso et al. (2016). A strong correlation was observed between pixel number and CL intensity (r = 0.97). The high correlation between the number of pixels and the CL intensity was expected since the intensity is related to the velocity of the flow present in the wall of the corpus luteum. Likewise, the number of colored pixels and total pixel intensity were positively correlated with vascular perfusion and P4 concentrations in mares (Romano et al., 2015) and ewes (Figueira et al., 2015). The intensity gives us more information about the characteristics of blood flow and can be used as a more sensitive complimentary analysis to increase the accuracy of the exam.

The CL diameter on day 7 after AI also showed a positive, albeit weak, correlation with the intensity of follicle pixels and did not correlate with the number of follicular pixels. This fact allows us to speculate on the application of such a characteristic in the complementary assessment of blood flow in ovarian structures. The idea would be to develop a more practical way of evaluating vascular perfusion than the pixel count to enable the use of this tool in the prediction of fertility in cattle.

According to Acosta (2007), the images of the POF in the spectral mode Doppler also revealed larger areas of flow and greater total mean velocity (TAMV), which were temporally correlated with the increase in plasma concentrations of estradiol and the luteinizing hormone peak. This also demonstrated functional relationships with the amount and speed of flow. The spectral mode, despite allowing a quantitative analysis of the flow velocity during the exam, undergoes drastic changes in these values due to changes in the angle of insonation (Ginther, 2014).

The increase in the blood flow intensity had a positive effect on the size of the POF and the CL, since this variable was statistically different and increasing for the SF, MF, and LF groups, respectively. Therefore, the follicle blood flow intensity did not differ between groups of pregnant and non-pregnant cows ($P>0.05$), in disagreement with Silva et al. (2006), who found higher systolic velocity and TAMV in follicles of mares that became pregnant. In addition to the possible variations between species, changes in the moment of ultrasound evaluation, that is, pre or post ovulation, may have contributed to this difference.

The intensity of blood flow in the CL did not differ significantly between pregnant and non-pregnant cows, but again, this could relate to the timing of when cows were examined, seven days after AI, suggesting that they may have corpora lutea at different phases.

Varughese et al. (2017) associated the number of lighter shades of color in the CL flow on days 12 and 21 of the cycle with the presence of turbulence and the occurrence of luteolysis, which highlights the importance of differentiating increases in flow intensity from the presence of turbulence.

5. Conclusions

The vascular perfusion of the preovulatory follicle and corpus luteum of Nellore cows subjected to timed artificial insemination varies according to the diameter of these structures. Additionally, follicle and corpus luteum size interfere with ovulation rate and progesterone production, respectively, but without affecting pregnancy rate.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: R.P. Barbero and M.R.B. Mello. Data curation: S.R.B. Couto, J.P.N. Andrade and M.R.B. Mello. Formal analysis: S.R.B. Couto and J.P.N. Andrade. Investigation: S.R.B. Couto, R.C.L. Morais, J.C.F. Jacob and M.R.B. Mello. Methodology: S.R.B. Couto, Y.B. Guerson, R.C.L. Morais, G.F. Grillo and J.P.N.

Andrade. Project administration: M.R.B. Mello. Resources: M.R.B. Mello. Supervision: M.R.B. Mello. Validation: J.P.N. Andrade, J.C.F. Jacob, R.P. Barbero and M.R.B. Mello. Visualization: R.C.L. Morais, G.F. Grillo, J.P.N. Andrade, J.C.F. Jacob, R.P. Barbero and M.R.B. Mello. Writing – original draft: S.R.B. Couto and Y.B. Guerson. Writing – review & editing: M.R.B. Mello.

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