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REGULAR ARTICLES

Correlations of corpus luteum blood flow with fertility and progesterone in embryo recipient mares

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Abstract

The aim of this study was to evaluate the correlation between the corpus luteum vascularization with the concentration of progesterone and the fertility of embryo recipient mares. Mangalarga Marchador mares (n = 33) were distributed into groups according to the days (D) after ovulation, as follows: D3 (n = 8), D4 (n = 8), D5 (n = 9), and D6 (n = 8). The evaluations of the corpus luteum, endometrium, and blood collection to quantify the progesterone concentration were carried out on D3, D4, D5, and D6. Among the parameters evaluated, only progesterone concentration on D6 differed from the other groups (P <0.05). A positive correlation (P <0.05) between the diameter and the area of the corpus luteum, and the objective and subjective methods of the corpus luteum vascular perfusion, was identified. Likewise, a positive correlation (P <0.05) was observed between the objective and subjective methods of the vascular perfusion in the corpus luteum and the progesterone concentration. The pregnancy rate obtained in this study (54.54%) was not affected (P> 0.05) by the day of embryo transfer, whose percentages were 37.50% (3/8) on D3, 50% (4/8) on D4, 66.70% (6/9) on D5, and 62.50% (5/8) on D6. It was estimated that with each increase on the day of embryo transfer, the pregnancy rate increases. The results allow to conclude that the corpus luteum vascularization in mares, evaluated by Doppler ultrasound, correlates with progesterone concentration and the embryo transfer day.

Keywords Doppler ultrasonography · Ovary · Progesterone · Reproduction · Vascularization

Introduction

Embryo transfer is a biotechnology that enables to select higher genetic merit mares to produce more than one foal per year. Furthermore, it can also be applied to mares that are in competition or training or having reproductive problems which prevents the pregnancy maintenance (Squires et al. 1999; Oliveira Neto et al. 2018). However, the success of this biotechnology depends on the perfect synchronization between donor and recipient animals, in addition to other factors like the corpus luteum action (Oliveira Neto et al. 2018; Ishak et al. 2017).

In equines, as stated by Ferreira-Silva et al. (2019), the accepted window of synchrony is to use recipient mares between +1 (ovulation 1 day before the donor) and −3 (ovulation 3 days after the donor) days from ovulation. Nonetheless, a high variation is observed in the pregnancy rates of recipient mares (Squires et al. 1999; Ferreira-Silva et al. 2018a, b). This variation reinforces the need for an accurate evaluation of the corpus luteum function.

The corpus luteum function is to produce progesterone, which prepares the endometrium to receive the embryo and to maintain the pregnancy (McCue et al. 1999; Wynn et al. 2018). Thus, ascertaining the progesterone concentration would be an efficient tool to estimate the functional activity of the corpus luteum (Bollwein et al.

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2002a, b). However, the adoption of this methodology in commercial embryo transfer programs is limited due to its high cost and low practicality.

The functional role of the corpus luteum has been evaluated by using B-mode ultrasonography. This tool provides images in real time of the follicular and corpus luteum development, allowing to assess its size, shape, and echogenicity (Ginther and Utt 2004). Recently, the mare's reproductive tract functionality was evaluated by color Doppler ultrasonography (Pugliesi et al. 2018; El-Shahat et al. 2020). The advantages of this approach are that it is not invasive, it is practical, and it is efficient (Ginther and Utt 2004; Ferreira et al. 2011).

Doppler ultrasonography, besides the morpho-echogenic features, allows to assess the vascular architecture and the hemodynamic aspects of vessels in various organs, including the corpus luteum (Ginther and Utt 2004; Ginther et al. 2007a, b). This evaluation can be performed based on both objective and subjective methods. In the first one, blood perfusion is stated by the number of colored pixels counted by a software. For the subjective one, tissue blood perfusion is estimated through the percentage of colored pixels observed in the image (Ginther and Utt 2004; Bollwein et al. 1998; Beltrame et al. 2017).

The relationship between luteal blood flow and progesterone production during the estrous cycle in mares was demonstrated by Bollwein et al. (2002a, b). These authors reported that corpus luteum vascularization is a reliable measure of the progesterone levels. However, to our knowledge, this evaluation has not yet been carried out considering the different days of the embryo transfer.

For this reason, it is hypothesized that the corpus luteum vascularization in mares, assessed by Doppler ultrasound, is related to the best day both to select recipient mares and to choose the best day for embryo transfer.

The objective of this work was to evaluate the correlation between the corpus luteum vascularization with the concentration of progesterone and the fertility of embryo recipient mares.

Materials and methods

Experimental animals and place

The trial was carried out in Fazenda Lagoa do Monteiro, Itabaiana, PB, Brazil, geographically situated at latitude 7° 19′ 44″ south and longitude 35° 19′ 58″ west.

During the breeding season, Mangalarga Marchador mares (*N*=33) were selected based on the body condition, which ranged from 5 to 7, considering a 1 to 9 scale, as described by Henneke *et al.* (1983). Additionally, other factors considered for selecting the mares included reproductive performance and clinical condition. Gynecological examination by ultrasonography was also used for identification of uterine fluid and endometrial alterations that could compromise fertility, as suggested by Taveiros *et al.* (2008). The animals' age ranged from 5 to 10 years, and the weighing ranged from 350 to 450 kg. These mares were raised on pasture based on Mombaça grass (*Panicum maximum*) and were given ad libitum access to water and mineral supplementation (Kromium®, Tortuga, Brazil).

Estrus induction and ovulation

The recipient mares were treated with intramuscular injection (IM) of 7.5 mg of dinoprost tromethamine (Lutalyse®, Zoetis, Brazil). Further, the ovaries were examined on a daily basis, using B mode ultrasonography, until the largest follicle reached the diameter of 33 mm. The uterine edema was scored as 2 (endometrial edema and clear endometrial folds) or 3 (endometrial edema and highlighted endometrial folds) as suggested by Figueiredo et al. (2011). At this time, ovulation was induced by injecting 1500 μg (IM) of deslorelin acetate (Sincrorelin®, Ouro Fino, Brazil). Ultrasound (B mode) examinations were carried out daily until the ovulation was identified.

According to the days after ovulation (D), the recipients were assigned to four groups, as follows: D3 (third day after ovulation), D4 (fourth day after ovulation), D5 (fifth day after ovulation), and D6 (sixth day after ovulation). Each group was composed of eight mares, except group D5 which had nine recipient animals. At the ovulation day, the corpus luteum and endometrium were evaluated by ultrasound examinations, and blood samples were collected to quantify the progesterone concentration.

Evaluation of ultrasonography parameters

Transrectal ultrasound examinations were performed by the same operator, using an ultrasound system (SonoScape®, model S2V, Co., Ltd., China) with a linear transducer, and an operating frequency of 5 to 10 MHz (SonoScape® Co., Ltd., China).

As proposed by Curran and Ginther (1995), the area (mm^2) and diameter (mm) of the corpus luteum were measured from a fixed image in ultrasonography B mode.

The subjective determination of the vascular perfusion of the corpus luteum was based on a video record of at least 60 s, from which the percentage of luteal tissue was estimated considering the color Doppler signals (Ginther and Utt 2004). Following guidelines according to Ginther and Utt (2004), endometrial vascularity was scored from 1 to 4. Regarding the objective evaluations, it was performed from three images with the highest number of colored signals in the luteal area using Adobe Photoshop 5.5 (Adobe Systems, San Jose, CA). The total number of pixels and their intensity were generated

by the ImageJ 1.31v program (National Institutes of Health, Bethesda, MD). The number of colored pixels was transformed into the vascularized luteal area considering one color pixel as 0.0465 mm^2.

The objective evaluation of the endometrium was performed by offline measurement of the area and the blood flow intensity (pixels), which provides a quantitative measure of the extent of perfusion and the relative speed (Ginther, 2007a). The recorded videos were converted and saved as GIF pictures. Five cross-sectional pictures of the middle segment of the horns and the uterine body were used to determine the area and intensity from each female. The average value was used for the analyses (Silva et al. 2005).

Progesterone analysis

Blood collection was performed by jugular vein venipuncture using a 4-mL Vacuette® tube coated with a clot activator (SiO_2) and a 25×8 needle. Blood samples were kept in an isothermal container (5°C), and they were immediately sent to the laboratory to be processed. The samples were then centrifuged at 4°C at 894 g for 20 min. Plasma was separated and frozen (−20°C) until P4 evaluation. The progesterone quantification was performed by the direct chemiluminescent technique (Immulite I® 103, Siemens Healthcare Diagnostics Ltd., Los Angeles, 104 CA, USA) with an analytical sensitivity of 0.2 ng/mL and accuracy of 106. Intra- and inter-assay variations were from 6.3 to 16% and from 5.8 to 16%, respectively.

Embryo transference and pregnancy diagnosis

The embryos collected were transferred to a petri dish containing culture medium (TQC—Nutricell, Nutrientes Ltda®, Campinas, SP, Brazil), and under a stereoscopic magnifying glass they were evaluated for their stage of development and their morphological quality. Only embryos in the blastocyst stage, classified between grades I and III, as described by McKinnon et al. (1993), were transferred to the recipient mares at day 3 (group D3), 4 (group D4), 5 (group D5), or 6 (group D6) post ovulation. The diagnosis of pregnancy was carried out on the 16th day after the embryo's transference by ultrasonography on day 25 after ET. All ultrasonography exams were performed by the same technician.

Statistical analysis

To determine the corpus luteum perfusion, the average from the results of the three images analysis was used. The percentage data were transformed into arcsine $\sqrt{P/100}$, and the results were expressed as mean ($\bar{x}$) ± standard error of the mean ($\sigma\bar{x}$). Further, the assumptions of normality (Shapiro-Wilk) and homoscedasticity (Lilliefors) were verified. For parametric and non-parametric variables, analysis of variance (ANOVA) followed by Tukey's test and the Kruskal-Wallis test followed by Dunn's test were applied, respectively.

To test the correlation between the variables, the data from all the animals, regardless of the group, were considered. Correlations were calculated using the Pearson correlation coefficient. To perform these analyses, the statistical program SPSS 16.0 was used, and a P value < 0.05 was taken as significant.

Multiple linear regression analysis, based on SAS 9.4 software, was used to evaluate the predictive power of the day of embryo transfer and subjective perfusion of the corpus luteum on the pregnancy rate. The multiple linear regression model was

$$y = \beta 0 + \beta 1x1 + \beta 2x2 + \ldots + \beta p{-}1xp{-}1 + \varepsilon$$

where y = dependent variable (PREG); $x1$, $x2$,..., xp-1 = independent variables (DAY and CLVP); $\beta 0$, $\beta 1$, $\beta 2$,..., βp-1 = regression parameters; ε = random error.

Results

The results from the progesterone quantification, endometrial and corpus luteum perfusion, and corpus luteum measurement are shown in Table 1. Among the parameters evaluated, only the progesterone concentration in the D6 group was significantly (P< 0.05) different when compared with the other ones.

Table 2 shows the correlation among the evaluated parameters. There was a positive and significant correlation (P< 0.05) between objective and subjective evaluation methods of the corpus luteum vascular perfusion. Likewise, a positive correlation (P< 0.05) was observed between the objective and subjective methods with the progesterone concentration. In addition, a positive correlation (P< 0.05) was found between the diameter and the area of the corpus luteum.

The pregnancy percentage obtained here (54.54%) was not affected by the day of embryo transfer (P> 0.05). According to the treatments, the pregnancy percentages obtained were 37.50% (3/8) on D3, 50% (4/8) on D4, 66.70% (6/9) on D5, and 62.50% (5/8) on D6.

The coefficient of determination (R^2) retrieved from the multiple regression model pointed that 89% of the pregnancy variation can be explained by the subjective perfusion of the corpus luteum and the day of embryo transfer.

The multiple linear equation model adjusted for the pregnancy rate was $\hat{Y}''$=64.33333+19.99227 × DT − 2.07285 × PSCL, where $\hat{Y}$ is the pregnancy rate, DT is the day of transfer, and PSCL is the subjective perfusion of the corpus luteum. Based on this regression, it was estimated that with each increase on the day of embryo transfer, the pregnancy rate

Table 1 Average values ($\bar{x} \pm \sigma\bar{x}$) of progesterone concentration, corpus luteum dimensions, vascular perfusion in the corpus luteum, and endometrium of recipient mares at the day of embryo transfer.

Parameters	Day of embryo transfer after ovulation			
	D3	D4	D5	D6
Corpus luteum area (mm^2)	10.24±0.7[a]	7.6±0.75[a]	7.4±0.72[a]	7.9±1.29[a]
Corpus luteum diameter (mm)	35.13±0.97[a]	31.38±1.48[a]	30.83±1.58[a]	31.21±2.27[a]
Subjective vascular perfusion in the corpus luteum (%)	42.29±9.23[a]	43.44±3.71[a]	49.64±2.55[a]	57.52±2.17[a]
Subjective vascular perfusion in the endometrium (1–4)	2.66±0.33[a]	2.25±0.25[a]	1.92±0.27[a]	1.33±0.21[a]
Objective vascular perfusion in the corpus luteum (mm^2)	612±131[a]	905±153[a]	1059±95[a]	1084±267[a]
Objective vascular perfusion in the endometrium (mm^2)	145.71±35.89[a]	196±49.9[a]	206.9±74.02[a]	288.24±22.56[a]
Progesterone concentration (ng/mL)	7.49±3.07[a]	6.49±1.37[a]	9.63±1.35[a]	20.67±3.37[b]

Variables with different letters in the same row are statistically significant ($P< 0.05$)

D3 3 days after ovulation, *D4* 4 days after ovulation, *D5* 5 days after ovulation, *D6* 6 days after ovulation

increases, unlike the subjective perfusion of the corpus luteum (Table 3).

The coefficient of determination (R^2) retrieved from the multiple regression model pointed that 89% of the pregnancy variation can be explained by the subjective perfusion of the corpus luteum and the day of embryo transfer.

The multiple linear equation model adjusted for the pregnancy rate was $\hat{Y}''=64.33333+19.99227 \times DT - 2.07285 \times CLVP$, where $\hat{Y}$ is the pregnancy rate, DT is the day of transfer, and CLVP is the subjective perfusion of the corpus luteum. Based on this regression, it was estimated that with each increase on the day of embryo transfer, the pregnancy rate increases, unlike the subjective perfusion of the corpus luteum (Table 3).

Discussion

Based on the ultrasonography, it is possible to characterize the vascular perfusion and estimate the function of reproductive organs and tissues throughout the animal's estrous cycle. This technique in association with other methodologies (e.g., hormonal measurements) allows for a better diagnosis, monitoring, and predictive abilities of reproduction in mares (Ginther et al. 2007a, b; Ferreira et al. 2011).

The progesterone levels quantified in the recipient mares with functional corpus luteum agree with those reported by Allen (2001) and Arruda et al. (2001), regardless of the ovulation day. It reinforces that the corpus luteum, already from the third day after ovulation, had the functional capacity to maintain the pregnancy until the placenta assumes its function. The highest progesterone concentration on D6 was not only expected as it corroborates the reports from Bollwein et al. (2002a, b) and Ginther et al. (2007a, b, 2008). These authors reported that the progesterone synthesis reaches the highest concentration on D5 and remains constant until the eleventh day after ovulation. According to the same authors, this is due to a rapid proliferation of the endothelial cells that establish a dense network of capillaries needed for the synthesis and secretion of progesterone.

Table 2 Pearson's correlation coefficients (r) among ultrasonographic parameters of the uterus and corpus luteum, as well as progesterone concentration from recipient mares at the day of embryo transfer

Correlated parameters	r
Corpus luteum diameter vs corpus luteum area	0.98*
Corpus luteum diameter vs objective vascular perfusion in the corpus luteum	−0.03
Corpus luteum diameter vs subjective vascular perfusion in the corpus luteum	−0.06
Corpus luteum diameter vs progesterone concentration	0.12
Corpus luteum area vs progesterone concentration	−0.12
Objective vascular perfusion in the corpus luteum vs subjective vascular perfusion in the corpus luteum	0.72*
Objective vascular perfusion in the corpus luteum vs progesterone concentration	0.54*
Subjective vascular perfusion in the corpus luteum vs progesterone concentration	0.63*
Objective vascular perfusion in the endometrium vs progesterone concentration	−0.26
Subjective vascular perfusion in the endometrium vs progesterone concentration	−0.33

*$P < 0.05$

Table 3 Effect of the day of embryonic transfer and subjective perfusion of the corpus luteum on the pregnancy rate based on multiple linear regression

DT	CLVP	TP	Multiple linear regression [1]		
3	42.29	37.50	Equation parameters	Estimated values	P-value
4	43.44	50.00	Intercept	64.33±10.289	<0.01
5	49.64	66.70	DT	19.99±2.144	<0.01
6	57.52	62.50	CLVP	−2.07±0.396	<0.01

$\hat{Y}$ pregnancy rate, *DT* day of embryo transfer, *CLVP* subjective perfusion of the corpus luteum, *TP* pregnancy rate

[1] $\hat{Y} = 64.33 + 19.99 \times \text{DT} - 2.07 \times \text{CLVP}$; $R^2 = 0.89$

In the current study, capillary proliferation was identified based on the correlation between progesterone concentration and the vascular perfusion in the corpus luteum, by applying both the objective and subjective methods. It is also possible to assume that the functional activity of the corpus luteum does not depend on its size since there was no correlation between the area of the corpus luteum and the progesterone concentration, which was also reported by Lüttgenau et al. (2011). Although Brogan et al. (2016) reported a correlation between these parameters, Herzog et al. (2010) highlighted that the blood flow is a better biomarker for progesterone concentration than the size of the corpus luteum.

Considering that the day of ovulation was different among the recipient mares, it was believed that the vascular perfusion in the corpus luteum was directly proportional to the day of ovulation, as well as to the progesterone concentration. However, these correlations were not identified here, suggesting that a functional corpus luteum has the same vascularization from its initial development stage to the beginning of its plateau phase, as discussed by Ginther et al. (2007a, b). For this reason, it is possible to hypothesize that this vascularization remains unchanged during the diestrus.

Regarding the objective and subjective evaluation methods, it is important to highlight that both of them were correlated and showed similar results for each tested group. Likewise, these evaluations were correlated with progesterone concentration, which agrees with the results reported by Bollwein et al. (2002a, b) and Ginther et al. (2007a, b). Therefore, the results reported here reinforce the application of the subjective evaluation method as an efficient tool to select recipient mares for embryo transfer.

The vascularization of the teak cells into the preovulatory follicles promotes the multiplication and nutrition of these cells to produce progesterone after the corpus luteum formation (Robinson et al. 2009; Araújo et al. 2013). Both vascularization and corpus luteum dimensions affect the progesterone production, mainly in females with a longer functional corpus luteum (Yan et al. 1998; Arashiro et al. 2010). However, in the present study, the area and diameter of the corpus luteum were not different between the experimental groups and they were not correlated. These results suggest that morphometric parameters are not accurate to estimate the progesterone production, as previously described by Veronesi et al. (2002).

Progesterone prepares the uterine environment to receive the embryo and to maintain the pregnancy (Vanderwall et al. 2016; Wynn et al. 2018). Thus, it was believed that during the functional phase of the corpus luteum there would be greater vascular perfusion in the endometrium. The increased vascularization would result in a higher progesterone action in the uterus, mainly in females with longer functional corpus luteum. Nonetheless, the endometrial vascular perfusion remained constant and did not support the hypothesis designed here. The decreased endometrial vascularization from day 5 of the functional activity of the corpus luteum, followed by an increase between the 10th and 13th days, was reported by Bollwein et al. (1998), Ginther et al. (2007a, b), and Ignácio et al. (2012).

Although the progesterone level is important for a successful embryo transfer (Vanderwall et al. 2016; Wynn et al. 2018; Hayes and Ginther 1986), it was not observed in the pregnancy rates from the mares with a longer luteal phase (D6). This finding suggests that the progesterone concentration from 6.49 ng/mL, as measured in the mares on D4, is likely enough to prepare the uterus to receive the blastocyst embryo and to maintain the pregnancy.

Based on the results, it is possible to conclude that the corpus luteum vascularization in mares, evaluated by Doppler ultrasound, correlates with the progesterone concentration and the embryo transfer day. Therefore, Doppler ultrasound is an important tool to select embryo recipient mares.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Statement of animal rights This study was approved by the animal ethics committee of the Federal Rural University of Pernambuco, CEUA-UFRPE (protocol number 011/2013).

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