UNIVERSIDADE DE SÃO PAULO
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

KARINE GALHEGO MORELLI

Use of Doppler ultrasonography for selection of recipients in embryo transfer programs in horses

São Paulo
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Use of Doppler ultrasonography for selection of recipients in embryo transfer programs in horses

VERSÃO CORRIGIDA

Dissertation submitted to the Postgraduate Program in Animal Reproduction of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Master's degree in Sciences.

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Advisor:

Prof. Guilherme Pugliesi, Ph.D.

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CERTIFICADO

Certificamos que a proposta intitulada "Uso da ultrassonografia Doppler na seleção de receptoras em programas de transferência de embriões em equinos", protocolada sob o CEUA nº 3575161020 (ID 008555), sob a responsabilidade de **Guilherme Pugliesi** *e equipe; Karine Galhego Morelli* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 09/12/2020.

We certify that the proposal "Use of Doppler ultrasonography for selection of recipients in embryo transfer programs in horses.", utilizing 150 Equines (150 females), protocol number CEUA 3575161020 (ID 008555), under the responsibility of **Guilherme Pugliesi** *and team; Karine Galhego Morelli* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 12/09/2020.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 11/2020 a 03/2022 Área: Reprodução Animal

Origem:	Animais de proprietários						
Espécie:	Equídeos	sexo:	Fêmeas	idade:	4 a 15 anos	N:	150
Linhagem:	Brasileiro de Hipismo e Bretão			Peso:	350 a 950 kg		

Local do experimento: O experimento será realizado no Pólo Regional Alta Mogiana Colina - APTA Regional, localizado em Colina - SP, na Avenida Rui Barbosa s/nº, região rural.

Comentário da CEUA: O experimento será desenvolvido no Polo Regional Alta Mogiana que faz parte do APTA, que se manifestou favorável ao projeto conforme TCLE assinado em anexo. Por questões relacionadas ao Cronograma do experimento, o projeto está sendo submetido apenas à CEUA da FMVZ/USP.

São Paulo, 21 de janeiro de 2021

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

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EVALUATION FORM

Author: MORELLI, Karine Galhego

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Date: 17/03/2023

Committee Members

Prof. Dr. Guilherme Pugliesi

Institution: Universidade de São Paulo – USP/FMVZ

Decision: ____

Prof. Dr. Jair Camargo Ferreira

Institution: Universidade de Franca - Unifran

Decision: ____

Prof. Dr. Júlio Cesar Ferraz Jacob

Institution: Universidade Federal Rural do Rio de Janeiro

Decision: ____

I dedicate this dissertation to my dear grandmother Joana (in memoriam), my heart out of my chest, to her I owe my essence, my love and all the meaning and reason of my life, to my father Leonardo (in memoriam), my biggest supporter in veterinary medicine and in the horse world, and to the great loves of my life, my mother Creuza, my sister Vanessa and my heart brother José Antonio (Zem), for dreaming my dreams, for not measuring efforts for me, for loving me unconditionally and for always being by my side supporting me. To you, all my love and gratitude.

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"There are many assumptions in science that are wrong. That's perfectly acceptable, they're the opening to find the ones that are right."

- *Carl Sagan*

RESUMO

MORELLI, K. G. **Uso da ultrassonografia Doppler na seleção de receptoras em programas de transferência de embriões em equinos.** 2023. 53p. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Pirassununga, 2023.

Objetivamos avaliar o impacto das características do corpo lúteo (CL) e do útero acessadas pela ultrassonografia em modo-B e Doppler-colorido em éguas receptoras no momento da transferência de embriões (TE) sobre os resultados da prenhez em um programa de TE. Éguas receptoras (n= 110), entre dias 3 a 9 após a ovulação espontânea, receberam um embrião fresco. Imediatamente antes da TE, o sistema reprodutor foi avaliado por palpação transretal quanto aos parâmetros a seguir: tônus uterino (0-3), ecogenicidade do CL (0-6), tipo de CL (homogêneo, trabeculado ou centro anecoico), área lútea (cm^2), ecogenicidade uterina (0-3), edema uterino (0-3), ecotextura uterina (0-3), perfusão sanguínea luteal (0-100%) e perfusão sanguínea uterina (1-4). Adicionalmente, uma amostra de sangue foi coletada por punção da veia jugular para dosagem plasmática de P4. Restrospectivamente, as receptoras foram classificadas de acordo com a área luteal (pequeno [≤ 6 cm^2] ou grande [> 6 cm^2]), perfusão sanguínea luteal (baixa [≤ 55%] ou alta [> 55%]) e concentração plasmática de P4 (baixa ≤ 9,98 ng/mL ou alto > 9,98 ng/mL). O diagnóstico de gestação foi realizado aos 15 e 30 dias de gestação. A perfusão sanguínea luteal foi significativamente maior (*P*= 0,03) em receptoras gestantes (n= 83) do que em receptoras não-gestantes (n= 27). A P/TE foi estatisticamente superior (*P*≤ 0,02) em éguas com perfusão sanguínea luteal e concentração plasmática de P4 altas. A perfusão sanguínea luteal foi o preditor significativo (*P*= 0,01) mais adequado da prenhez em comparação com a área luteal e concentração plasmática de P4. Adicionalmente, somente a perfusão sanguínea luteal apresentou efeito linear (*P*= 0,03) e cúbico (*P*= 0,004) sobre a P/TE. Em conclusão, a perfusão sanguínea do CL determinada pelo Doppler-colorido pode ser utilizada em tempo real para selecionar as receptoras com maiores chances de manter a prenhez em programas de TE em equinos.

Palavras-chave: Corpo lúteo. Perfusão sanguínea. Doppler colorido. Éguas receptoras.

ABSTRACT

MORELLI, K. G. **Use of Doppler ultrasonography for selection of recipients in embryo transfer programs in horses.** 2023. 53p. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Pirassununga, 2023.

We aimed to evaluate the impact of corpus luteum (CL) and uterine characteristics accessed by B-mode and color Doppler ultrasonography in recipient mares at the time of embryo transfer (ET) on pregnancy outcomes. Recipient mares (n=110), between days 3 to 9 after spontaneous ovulation, received a fresh embryo. Immediately before ET, the reproductive system was assessed by transrectal palpation for the following parameters: uterine tone (0-3), CL echogenicity (0-6), CL type (homogeneous, trabecular or anechoic center), luteal area (cm2), uterine echogenicity (0-3), uterine edema (0-3), uterine echotexture (0-3), luteal blood perfusion (0-100%) and uterine blood perfusion (1-4). Additionally, a blood sample was collected by puncture of the jugular vein for plasma P4 dosage. Retrospectively, recipients were classified according to the luteal area (small [≤ 6 cm2] or large [> 6 cm2]), luteal blood perfusion (low [≤ 55%] or high [> 55%]), and plasma concentration of P4 (low ≤ 9.98 ng/mL or high > 9.98 ng/mL). Pregnancy diagnosis was performed at 15 and 30 days of gestation. Luteal blood perfusion was significantly higher (P= 0.03) in pregnant recipients (n= 83) than in non-pregnant recipients (n= 27). Overall P/ET was higher (P≤ 0.02) in mares with high luteal blood perfusion and high P4. Luteal blood perfusion was the most adequate significant (P= 0.01) predictor of pregnancy compared with the luteal area and plasma P4 concentration. Only luteal blood perfusion showed a linear (P= 0.03) and cubic (P= 0.004) effect on P/ET. In conclusion, CL blood perfusion determined by color-Doppler can be used in real-time to select recipients with the greatest chance of maintaining pregnancy in equine ET programs.

Keywords: Corpus luteum. Blood perfusion. Color Doppler. Recipient mares.

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SUMMARY

1 INTRODUCTION

The embryo transfer (ET) is a reproductive biotechnique widely used in the equine industry to increase the number of foals generated from superior mares during one breeding season and to overcome several issues related to subfertility (ARRUDA et al., 2001). The recipients play a fundamental role in pregnancy success after ET (SQUIRES et al., 1999; ARRUDA et al., 2001; PANZANI et al., 2016). Selection of recipients at the time of ET is a major challenge and the most critical point before ET (SQUIRES et al., 1999; VANDERWALL & WOODS, 2007; MCKINNON & SQUIRES, 2007). Therefore, to enhance the chance for selection of a female with a high potential for pregnancy maintenance it is recommended to have at least three recipients available for each embryo's donor (ARRUDA et al., 2001; MCKINNON & SQUIRES, 2007).

Several characteristics are evaluated in recipients during the selection process. Around the period of the donor's ovulation, the recipients in estrus are assessed every 24 or 48 h for determination of ovarian follicular growth, ovulation, and characteristics of the uterus (VANDERWALL & WOODS, 2007). On the day of the ET, the recipients with ovulations detected one day before to five days after the moment of the donor's ovulation are evaluated and selected based on physical examination of the female reproductive tract through transrectal palpation and B-mode ultrasonography. The main characteristics observed in a suitable recipient are the presence of a corpus luteum (CL); firm and closed cervix; a uterus with high tone, homogeneous morpho-echogenicity, absence of fluid or abnormality; and absence of any vaginal or vulvar abnormality (CARNEVALE et al., 2000; ARRUDA et al., 2001).

Most of the uterine and cervical characteristics evaluated are related to the progesterone (P4) effects. Thus, the presence of an active CL, which has the main function to produce P4 is crucial to prepare the endometrium to receive the embryo and to maintain pregnancy (GINTHER et al., 2007; FERREIRA et al., 2018; WYNN et al., 2018). Determining the circulating P4 concentrations would be an efficient tool to estimate the CL activity (BOLLWEIN et al., 2002); however, its high cost and difficulty in obtaining results in real-time make its use limited (SALES et al., 2021). Also, the CL activity cannot be accurately assessed by transrectal palpation or B-mode ultrasound (KAHN, 1994; GINTHER, 1995), as morpho-echogenic characteristics are not associated with luteal functional status in horses (ARRUDA et al., 2001). In this regard, the selection of bovine recipients based only on CL size on the day of ET can result in the transfer of embryos to a non-receptive uterus with the presence of a poorly active or even non-functional CL (PUGLIESI et al., 2019).

Color-Doppler ultrasonography emerged as an alternative to assess the functional status of the CL in cattle (ACOSTA et al., 2005; SIDDIQUI et al., 2009; HERZOG et al., 2010; PUGLIESI et al., 2016), horses (GINTHER et al., 2014) and small ruminants (BALARO et al., 2017). Ferreira & Meira (2011) suggested that evaluation of CL and uterus by Doppler ultrasonography could be an auxiliary tool for the selection of recipient mares with a better P4 profile and uterine environment for maintaining the pregnancy. Thus, studies were carried out to evaluate CL and uterine blood perfusion before ET in recipient mares (BROGAN et al., 2016; FERREIRA et al., 2020; AZEVEDO et al., 2021; SALES et al., 2021), but the results were controversial and the restricted number of animals used (n=15 to 48) has limited powerful conclusions. In addition, all studies used colored pixel counts or colored areas to estimate blood perfusion, which is a time-consuming procedure.

Therefore, more powerful studies using practical methods to assess CL activity by Doppler imaging are needed in horses to understand the impact of luteal blood perfusion on uterine receptivity in ET programs. The present study aimed to characterize the impact of CL and uterine characteristics accessed by B-mode and color-Doppler ultrasonography in recipient mares at the time of ET on pregnancy outcomes in a large commercial ET program. We hypothesized that in recipient mares: 1) the CL blood perfusion has a greater impact than CL size on pregnancy rates after ET; and 2) a greater CL activity indicated by CL blood perfusion is positively associated with pregnancy rates.

2 MATERIALS AND METHODS

2.1 LOCATION AND MANAGEMENT

The experiment was conducted from November/2021 to May/2022 on a commercial farm located in Itaperuna-RJ, Brazil (Latitude 21°12′17″ S, Longitude: 41°53′16″ W, altitude of 123 m sea level). A total of 110 embryo-recipient mares of the Mangalarga Marchador breed were included in the experiment. The mares were kept under extensive rotational management in Tifton 85 paddocks, with free access to mineral salt and water. The study was approved by the Committee on Ethics in the Use of Animals of the School of Veterinary Medicine and Animal Science of the University of São Paulo (CEUA/FMVZ) at the meeting on 12/09/2020 under protocol CEUA nº 3575161020.

2.2 ANIMALS

Mangalarga Marchador mares between 3 and 26 years old (9.2 ± 0.88 years) and with a mean body condition score of 7 (scale of 1 to 9, HENNEKE et al., 1983) were used as embryo donors. The recipient mares used were also from the Mangalarga Marchador breed and aged between 3 and 16 years old (9.3 ± 0.28 years), weighted between 370 and 460 Kg (410.5 ± 4.29 Kg) and with a mean body condition score of 5 (scale of 1 to 9, HENNEKE et al., 1983) throughout the experiment.

Only recipient mares with the absence of significant clinical or reproductive issues were considered in the study. Mares presenting more than one functional CL, low cervical tone, intrauterine fluid, and/or uterine alterations that could compromise fertility on the day of ET were excluded. Finally, mares that received more than 3 embryos and did not become pregnant were not enrolled in the study.

2.3 EXPERIMENTAL DESIGN AND HANDLING OF RECIPIENT MARES

On the day of ET, the reproductive tract of all recipients within 3 to 9 days after ovulation was assessed through transrectal palpation and B-mode ultrasound by a single veterinarian. An average of 2 to 3 recipients were available for each donor. Recipients considered to have no apparently abnormal condition (low cervical or uterine tone, presence of edema or uterine fluid, cervical laceration, abnormal vulvar conformation, and free of any infectious diseases) were submitted to a new blind assessment by a second evaluator. In this exam, recipients were evaluated by transrectal palpation and B and color-Doppler ultrasonography, and a blood sample was collected for P4 assay. Within a maximum of 1 hour after the last assessment and according to the viability of fresh embryos collected on that day, a grade I or II embryo (n= 110) was transferred by a single operator (MCKINNON & SQUIRES, 1988). Therefore, the palpation and ultrasonography characteristics determined by B and Color modes determined by the second evaluator were used retrospectively to analyze the impact of these factors on pregnancy success but not for selecting the 110 recipients that received the ET.

2.4 TRANSRECTAL PALPATION AND B-MODE ULTRASONOGRAPHY

For the evaluations performed by transrectal palpation, the cervical tone was assessed using score grades ranging from 1 to 3 (open to closed) and uterine tone ranging from grade 0 to 3 (minimum to maximum) in one-half increments (CUERVO-ARANGO & NEWCOMBE, 2008).

For B-mode ultrasonography, recipients were evaluated after the transrectal palpation using a Duplex ultrasound equipment (MyLab™ DeltaVET device; Esaote Healthcare, Italy) coupled to a multifrequency linear transducer. The ultrasound was maintained in the same pre-setting throughout the experiment (gain 100%, RES-A, frequency 6.3 MHz, Depth 67 mm X/M, C1, PRS1). By B-mode ultrasonography, the following uterine characteristics were evaluated: 1) uterine echogenicity (scored from 0 to 3; minimum to maximum echogenicity); 2) uterine echotexture (scored from 0 to 3; homogeneous to heterogeneous); 3) uterine edema (scored from 0 to 3; minimum to maximum) in medium increments for all evaluations. In addition, the CL was classified regarding the presence of cavity (homogeneous, trabeculated, or anechoic center), its echogenicity (scale from 0 to 6; minimum to maximum echogenicity), and luteal area (cm^2). The size of the luteal tissue was determined by subtraction of the total area of the CL and the area of trabeculae or the anechoic center, as adapted from Pierson & Ginther (1985) and Fleury et al. (2006).

Pregnancy diagnosis was performed by B-mode transrectal ultrasonography based on the visualization of an embryonic vesicle at 12 days of gestation and confirmation was performed through visualization of the embryo's heartbeat at 30 days.

2.5 COLOR-DOPPLER MODE ULTRASONOGRAPHY

The recipient mares were submitted to evaluations in color-Doppler mode performed by a single trained operator. The same ultrasound device was used with the following setting for color Doppler mode (gain 70%, PRF 740 Hz, frequency 6.3 MHz, PRC M/2, PRS 3), allowing the detection of blood flow from 5.4 cm/s.

Firstly, vascular characteristics in the endometrium and myometrium were assessed by detecting the color signals of blood flow, using the transrectal transducer over the medial portion of each uterine horn for 60 seconds of scanning cross-sectional images of each horn. Blood perfusion was estimated by real-time subjective scoring of the extent of colored areas within the endometrium and myometrium, as previously described in horses by Ginther & Utt (2004). Blood perfusion scores indicated null (1), minimum (2), intermediate (3), and maximum (4) perfusion.

For luteal blood perfusion determination, the CL was evaluated through real-time and subjective analysis, according to the proportion (0 to 100%) of luteal tissue area with color signals indicating blood perfusion during a 60-second continuous examination as described by Ginther & Utt (2004). This approach accounts for the whole scanning of the tissue, as the score is given based on a mental 3D reconstruction of the luteal tissue, and allows a real-time diagnosis and rapid decision-making.

2.6 BREEDING MANAGEMENT OF EMBRYO DONOR MARES

All donor mares received an intramuscular injection of 1 mg of deslorelin acetate to induce ovulation in the presence of a dominant follicle with a diameter ≥ 33 mm associated with uterine edema (CUERVO-ARANGO & NEWCOMBE, 2008). Ultrasound examination of the reproductive tract of donors was performed daily from the dominant follicle until the detection of ovulation (day 0). The mares were inseminated using fresh, chilled, or frozen semen according to the semen of each mating. For fresh and chilled semen, artificial insemination was performed 24 hours after the ovulation induction. For frozen semen, artificial insemination was performed within a maximum of 6 hours after ovulation detection.

The donors were submitted to the uterine flushing procedure for embryo collection, using the non-surgical method adapted from Squires et al. (1985). Harvests were performed between the 7th and 10th day after ovulation of the donor mare.

2.7 EMBRYO RECOVERY, EVALUATION, AND TRANSFER PROCEDURES

A total of 161 uterine flushing procedures were performed, where 127 embryos were recovered. After collection, embryos were evaluated in a stereo microscope with a magnification between 30 and 80X for classification according to morphology and quality (MCKINNON & SQUIRES, 1988). Only embryos classified as grade I (excellent; spherical, with uniform cell size, color, and texture) or grade II (good, with minor imperfections such as few extruded blastomeres, irregular shape, or trophoblast separation) were transferred to recipients. Therefore, a total of 110 embryos were transferred to recipients in normal conditions and were considered in this study.

2.8 BLOOD COLLECTION FOR PLASMA PROGESTERONE MEASUREMENT

Immediately before ET, blood samples were collected from the recipients through jugular vein puncture in 10 mL vacuum tubes containing sodium heparin. After collection, samples were centrifuged (1500 x g for 20 min; GINTHER et al., 2007) and stored at -20 ºC for subsequent P4 measurement. Plasma P4 concentration (ng/mL) was quantified at the Laboratory of Hormonal Dosages of the School of Veterinary Medicine and Animal Science of the University of São Paulo, using a radioimmunoassay kit MP Biomedicals (RIA, REF: 0717010-CF, P4, MP Biomedicals, LLC, EUA) following the manufacturer's protocol. The intra-assay CV for low and high controls and mean sensitivity were 4.2%, 1.9%, and 0.07 ng/mL, respectively.

2.9 STATISTICAL ANALYSES

Firstly, the CL, uterine characteristics, and P4 concentrations determined on the day of ET were compared between pregnant and non-pregnant recipients, between low and high blood perfusion groups, and between small and large CL size groups by ANOVA using PROC MIXED from SAS (version 9.2) and considering the fixed effects of gestational status (pregnant or non-pregnant), blood perfusion class or CL size class.

The binomial variable pregnancy rate was evaluated in two approaches using multivariate models by PROC GLIMMIX of SAS, considering the effects of all characteristics of CL, uterus, P4 concentration, and day of ovulation of the recipient. Thus, the CL, uterus, and P4 assessments were analyzed as categorical variables (small or large; high or low perfusion and high or low P4 concentration) in a multivariate statistical model, for this, a ROC curve was generated by the GraphPad Prism software 8 and was used to determine the class cutoff and area under the curve (AUC). Thus, recipient mares were retrospectively divided into subgroups according to CL area [small (≤ 6 cm^2) or large (> 6 cm^2)], CL blood perfusion (low [≤ 55%] or high [> 55%]), endometrial blood perfusion (low [≤ 2] or high [≥ 3]), myometrial blood perfusion (low [≤ 2] or high [≥ 3]) and plasma P4 concentration [low (≤ 9.98 ng/mL) or high (> 9.98 ng/mL)]. Also, the recipient's day of ovulation on the ET (3 to 9 days after ovulation) was considered in the model as a categorical variable. Thus, in the first model, the factors included were the type of semen (fresh, refrigerated, and frozen), day of release of the donor embryo (days 7 to 9), embryo quality grade (I or II), development stage embryonic (morula compact, morula, early blastocyst, blastocyst, and expanded blastocyst), day of ET (days 3 to 9), CL type (homogeneous, trabeculated or anechoic center), luteal area (small or large), myometrial and endometrial blood perfusion (low or high), cervical tone, uterine tone, uterine edema, uterine echogenicity, uterine echotexture, echogenicity of CL and plasma P4 concentration (high or low). Multivariate models were constructed and the stepwise backward elimination method was applied, considering the Wald criterion (factors with $P > 0.2$ are excluded from the model). Therefore, the final pregnancy rate (P/ET) models only included the effects of plasma P4 concentration class (high or low), CL blood perfusion class (high or low), CL size class (small or large), and the class of endometrial perfusion (high or low). In a secondary analysis, CL area and blood perfusion, and plasma P4 concentration were considered as continuous variables in individual statistical models using logistic regression by PROC LOGISTIC of SAS. According to the significant effects the procedure was used to determine whether the effect is linear, quadratic, or cubic. Significant and more complex models were selected.

Finally, we performed Pearson's correlation test to determine the correlations between luteal area, luteal perfusion, and P4 concentration and Fisher's exact test to assess whether pregnancy rate is dependent or independent of luteal blood perfusion and plasma P4 concentration.

Results were presented as means ± SEM. Probabilities of $P < 0.05$ indicate significant difference whereas probabilities of $0.05 > P < 0.1$ have a tendency toward significance.

3 RESULTS

3.1 OVARIAN AND UTERINE CHARACTERISTICS AND PLASMA P4 CONCENTRATIONS

Overall, the average CL area was 6.13 ± 0.16 cm^2, the average CL blood perfusion was 55 ± 0.5%, and the average plasma P4 was 9.52 ± 0.36 ng/mL. In pregnant animals, the average area was 6.12 ± 0.19 cm^2, the average blood perfusion was 57 ± 1.4% and the average plasma P4 concentration was 9.85 ± 0.43 ng/mL and in non-pregnant animals it was 6.18 ± 0.31 cm^2, 51 ± 1.7% and 8.50 ± 0.54 ng/mL respectively.

There was no significant difference ($P > 0.1$) between pregnant and non-pregnant recipient mares for plasma progesterone, transrectal palpation, and B-mode parameters (Table 1). However, a greater ($P = 0.04$) luteal blood perfusion was detected in pregnant recipients when compared to non-pregnant recipients (56.9 ± 1.40% and 50.9 ± 1.80%, respectively). There was also a trend towards higher P4 ($P = 0.07$) in pregnant recipients than in non-pregnant recipients.

Recipients were also classified based on the luteal blood perfusion category (high or low; Table 2) and on the luteal size category (small or large; Table 3). No significant difference ($P > 0.1$) was detected between classes of CL size or blood perfusion for uterine (endometrial and myometrial blood perfusion) and plasma P4 concentration. For luteal characteristics, the only significant difference was the expected greater luteal blood perfusion in the high perfusion category (Table 2) and the greater CL size in the large CL category compared to the small CL-class (Table 3).

Table 1. Mean ± SEM of tone, echogenicity, edema, and echotexture of the uterus, endometrial and myometrium blood perfusion, area, blood perfusion and echogenicity of the CL, and plasma P4 of pregnant and non-pregnant equine recipients evaluated by B and Color-Doppler ultrasonography at the time of ET.

End-points	**Pregnancy status**		
	Pregnant (n= 83)	**Non-pregnant** (n= 27)	***P*-value**
Uterine characteristics			
Tone	1.99 ± 0.01	2.00 ± 0.00	0.67
Echogenicity (0 – 3)	1.72 ± 0.04	1.74 ± 0.07	0.69
Edema (0 – 3)	0.34 ± 0.03	0.35 ± 0.05	0.52
Echotexture (0 – 3)	0.45 ± 0.03	0.41 ± 0.05	0.25
Endometrial blood perfusion (1 – 4)	1.88 ± 0.08	1.72 ± 0.13	0.43
Myometrium blood perfusion (1 – 4)	1.86 ± 0.07	1.76 ± 0.10	0.65
Corpus luteum characteristics			
Luteal echogenicity (0 – 6)	3.99 ± 0.09	3.92 ± 0.15	0.76
Luteal area (cm^2)	6.12 ± 0.19	6.18 ± 0.31	0.79
Luteal blood perfusion (%)	56.9 ± 1.40	50.9 ± 1.80	**0.04**
Plasma P4 (ng/mL)	9.85 ± 0.43	8.49 ± 0.54	**0.07**

Table 2. Mean ± SEM of luteal area, luteal blood perfusion, endometrial blood perfusion, myometrial blood perfusion and plasma P4 on the day of ET in equine recipients categorized into two subgroups according to luteal blood perfusion (low [≤55%] or high [>55%]).

End-points	**Luteal blood perfusion category**		
	Low	**High**	***P*-value**
Endometrial blood perfusion (1 – 4)	1.87 ± 0.09	1.81 ± 0.10	0.54
Myometrial blood perfusion (1 – 4)	1.84 ± 0.08	1.83 ± 0.09	0.96
Luteal area (cm^2)	6.29 ± 0.21	5.93 ± 0.24	0.26
Luteal blood perfusion (%)	47.0 ± 0.80	66.8 ± 1.10	**<0.0001**
Plasma P4 (ng/mL)	9.59 ± 0.45	9.42 ± 0.59	0.82

Table 3. Mean ± SEM of luteal area, luteal blood perfusion, endometrial blood perfusion, myometrial blood perfusion and plasma P4 on the day of ET in equine recipients categorized into two subgroups according to luteal area (small [≤6 cm^2] or large [>6 cm^2]).

End-points	**Luteal area category**		
	Small	**Large**	***P*-value**
Endometrial blood perfusion (1 – 4)	1.76 ± 0.09	1.96 ± 0.10	0.14
Myometrial blood perfusion (1 – 4)	1.75 ± 0.08	1.95 ± 0.09	0.12
Luteal area (cm^2)	5.02 ± 0.08	7.64 ± 0.21	**<0.0001**
Luteal blood perfusion (%)	55.8 ± 1.60	54.6 ± 1.60	0.63
Plasma P4 (ng/mL)	9.06 ± 0.43	10.13 ± 0.60	0.23

The correlation between the CL end-points and plasma P4 concentration on the day of ET is shown (Table 4). No significant correlations were detected between luteal blood perfusion and plasma P4 or luteal area, but a weak positive correction between luteal area and plasma P4 concentration was observed.

Table 4. Pearson correlation coefficients (r) were evaluated in relation to luteal area, luteal blood perfusion as well as plasma P4 concentration in embryo recipient mares at the time of ET.

Correlated variables	**r**	***P*-value**
Luteal area (cm^2) *vs.* Luteal blood perfusion (%)	-0.06	0.55
Luteal area (cm^2) *vs.* Plasma P4 (ng/mL)	0.21	**0.03**
Luteal blood perfusion (%) *vs.* Plasma P4 (ng/mL)	0.08	0.42

3.2 EFFECTS ON PREGNANCY RATES (P/ET)

A total of 83 out of the 110 ETs (75.5%) resulted in diagnosed pregnancies on day 30 after ET. No pregnancy losses were detected between the early (12 days) and late (30 days) diagnoses.

When all variables were used in the statistical model for the determination of factors affecting P/ET, any characteristic related to the embryo (age, quality, or semen source) or by the day of ET not significantly influenced the P/ET at day 30 ($P > 0.1$). The P/ET obtained according to the day of ET were: for day 3, 66.7% (2/3), day 4, 70.6% (12/17), day 5, 80.0% (20/25), day 6, 84.4% (27/32), day 7, 70% (14/20), day 8, 54.5% (6/11), and day 9, 100% (2/2).

The P/ET was not affected ($P > 0.1$) by the luteal area or uterine (endometrial or myometrial) blood perfusion (Figure 1). However, P/ET was affected by the CL blood perfusion class ($P = 0.007$) and plasma P4 concentration class ($P = 0.02$). A 35.8% increase in P/ET was observed in the high luteal perfusion group (41/46) compared to the low perfusion group (42/64); whereas a 26.1% increase in P/ET was observed in the group with high P4 concentration (41/48) compared to the group with low concentration of P4 (42/62).

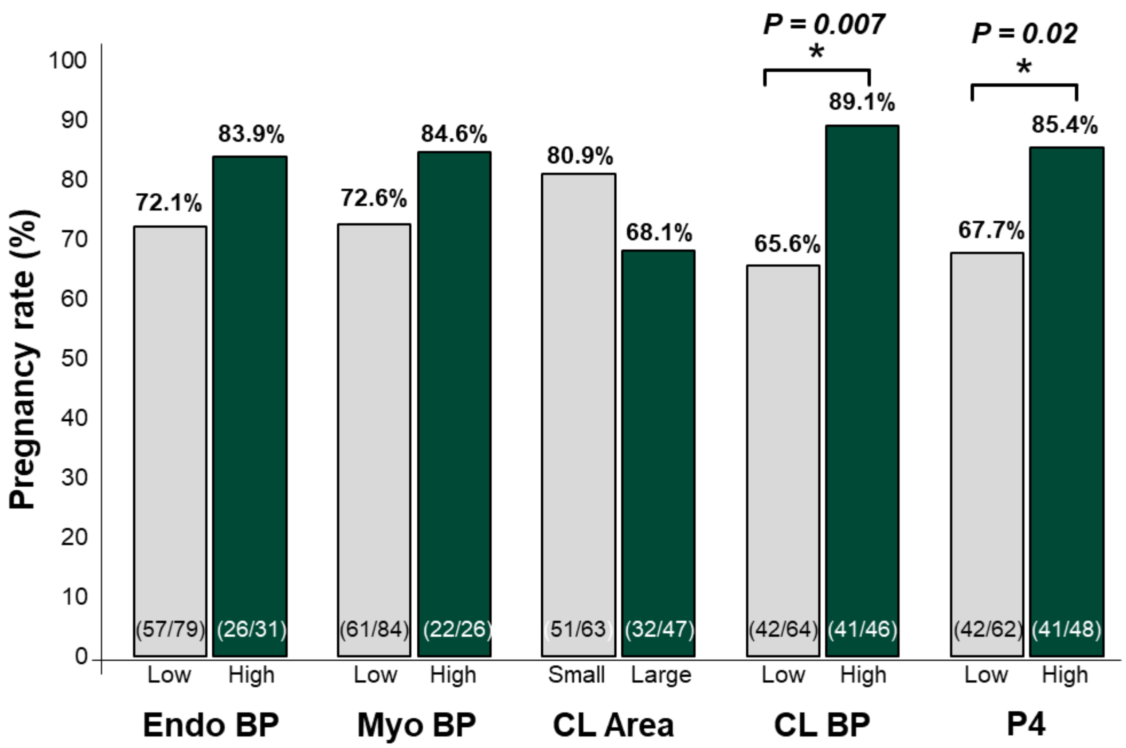

Figure 1. Pregnancy rate (P/ET; %) in recipients mares (n=110) divided into 2 groups according to endometrial blood perfusion (Endo BP; low [1 and 2] or high [3 and 4]); myometrial blood perfusion (Myo BP; low [1 and 2] or high [3 and 4]) CL area (small [≤ 6 cm^2] or large [> 6 cm^2]); CL blood perfusion (CL BP; low [≤ 55%] or high [> 55%]) and plasma P4 concentrations (P4; low [≤ 9.5 ng/mL] or high [> 9.5 ng/mL]). P/ET was significantly affected ($P = 0.007$) by CL BP class and P4 concentration ($P = 0.02$), as indicated by an asterisk (*).

Analysis of the ROC curve indicated a cutoff point of 5.94 cm^2 for CL area, 52.5% for CL blood perfusion, and 9.98 ng/mL for plasma P4 concentration.

When these characteristics were used as predictors of pregnancy success, the analysis indicated that only luteal blood perfusion (AUC= 0.66) was a significant (*P*= 0.01) predictor of pregnancy in mares at the time of ET when compared with CL area (AUC= 0.53) and plasma P4 concentration (AUC= 0.60) (Figure 2).

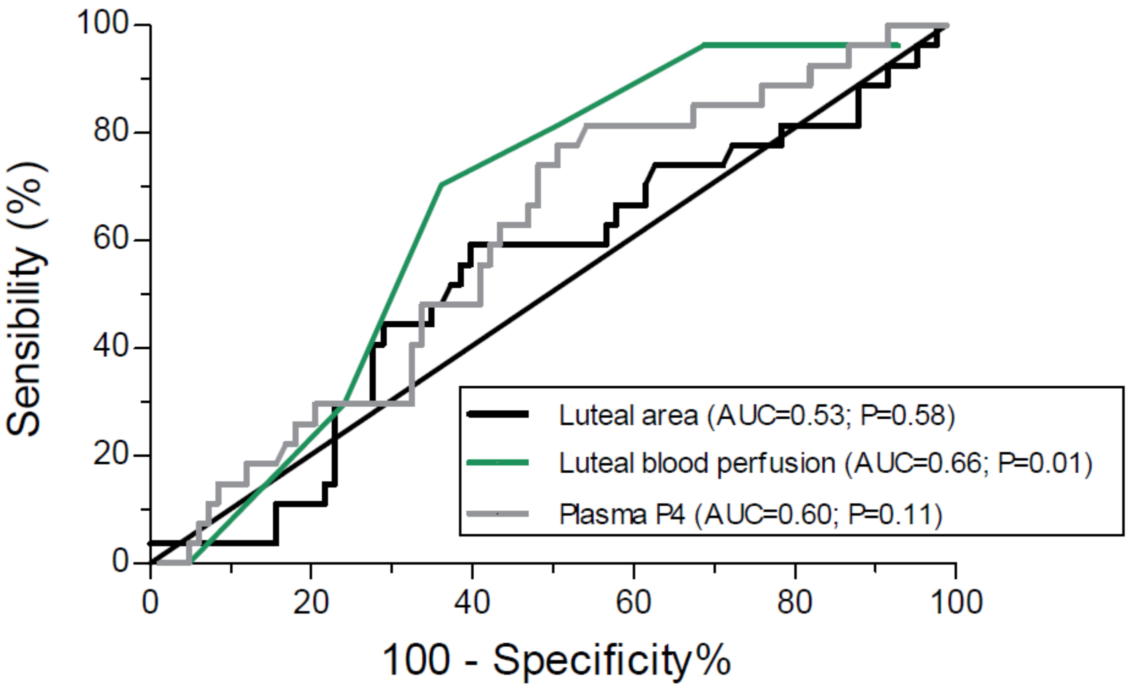

Figure 2. Receiver operating characteristic (ROC) curves of the three pregnancy predictors on the day of ET. The horizontal and vertical axes are a false positive rate (1 - specificity) and sensitivity, respectively. The luteal blood perfusion provided the most appropriate prediction of pregnancy when compared to the CL area and plasma P4 concentration.

Finally, recipients were separated into four different groups according to possible combinations of the two classes of CL blood perfusion and two classes of P4 concentration (High CLBP and High P4; High CLBP and Low P4; Low CLBP and High P4, Low CLBP and Low P4). The P/ET differed among the groups (*P*= 0.002), as indicated by the greater P/ET in the groups of high CLBP/high P4 and high CLBP/low P4 compared to animals with low CLBP/ low P4.

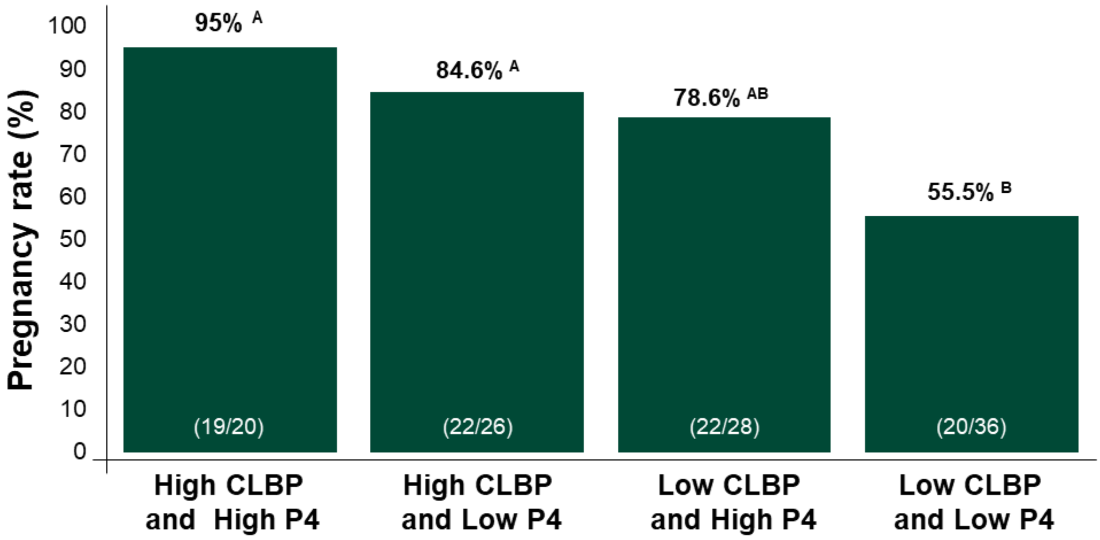

Figure 3. The pregnancy rate in relation to corpus luteum blood perfusion (high CL BP or low CL BP) and P4 concentration (high P4 or low P4) classes. $^{A, B}$ Means with different superscripts were considered using Fisher's test (P< 0.05).

When luteal area, CL perfusion, and plasma P4 concentration were evaluated as a continuous variable, CL area and P4 concentration did not affect P/ET (P> 0.1). A linear (P= 0.03) and cubic (P= 0.004) effects were detected for blood perfusion of CL, indicating the positive relationship between luteal blood perfusion at the time of ET with the probability of pregnancy (Figure 3A). However, the cubic effect indicated that P/ET is negatively associated with CL blood perfusion up to 45%, followed by a positive relationship up to 75% and then a negative relationship up to 85% (Figure 3B).

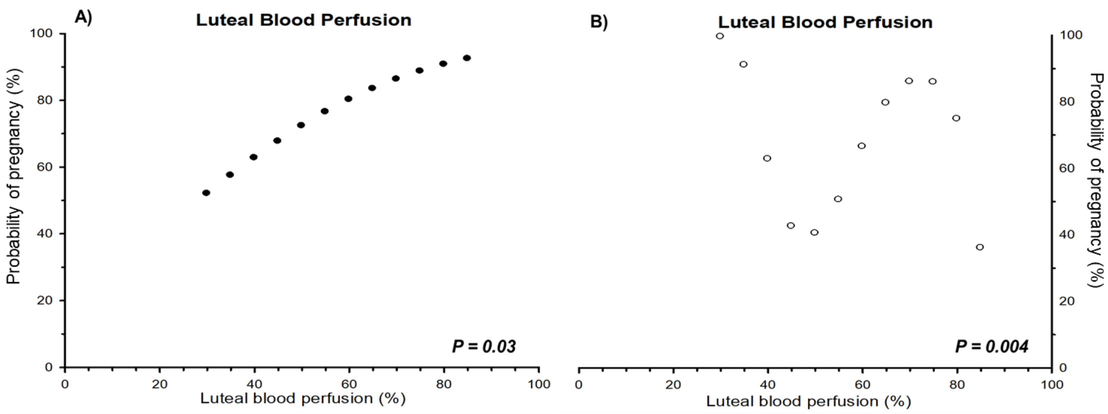

Figure 4. Probability of pregnancy according to a linear (panel A) or cubic (panel B) relationship with the proportion of luteal signals indicating blood perfusion in recipient mares at the time of ET (n=110). (A): Probability of pregnancy = exp (0.0439x -1.2366)/1 + exp (0.0439x -1.2366). (B): Probability of pregnancy = exp (-0.00032x^3 + 0.0579x^2 – 3.3498x + 62.3524)/ 1 + exp (-0.00032x^3 + 0.0579x^2 – 3.3498x + 62.3524); P< 0.05.

4 DISCUSSION

The present study is the first to show that it is possible to select more assertively and in real-time, embryo recipients through luteal blood perfusion at the time of ET in horses. Revealing that the greater the luteal blood perfusion, the greater the probability of pregnancy and reporting an almost 36% increase in P/ET when we added CL color Doppler assessment into the ET routine in previously selected mares. Thus, under the current conditions of our study, luteal blood perfusion on the day of ET was the most important characteristic that affected the maintenance of pregnancy in embryo recipient mares.

We know that the selection of recipients is one of the most important factors that affect the efficiency of an ET program in horses. Veterinarians in commercial ET programs usually select recipients by transrectal palpation and ultrasonography in B-mode. However, to estimate the function of reproductive organs and to improve the prediction of a receptive uterine environment, other diagnostic methods need to be associated, such as hormone dosages and vascular perfusion of the reproductive tract (GINTHER et al., 2007; FERREIRA et al., 2011).

The pregnancy establishment after ET can be influenced by several factors related to the embryo and recipient. Regarding those related to the embryo, embryonic quality, age, size, degree of development, and preservation are the most critical factors associated with gestational failure. In the present study, the first analysis of pregnancy rates after ET was performed with all the above-mentioned factors, but none of them affected P/ET. This result was somehow expected as there was a pre-selection to use only grade I and II embryos in order to optimize the P/ET. In addition, only fresh embryos were used, which are the embryos with the greatest chances to develop and result in a successful pregnancy in horses (SQUIRES & SEIDEL, 1995; SQUIRES, 2006). Previous studies (CARNEY et al., 1991; SQUIRES, 1993; FLEURY & ALVARENGA, 1999; JACOB et al., 2012) have also indicated that the age and size of the embryo do not influence the P/ET when embryos from 6 to 10 days after fertilization are transferred in an adequate handling and transfer techniques.

In addition to embryo-related factors, several other factors inherent to the recipient influence the P/ET, such as age, reproductive history, degree of synchrony between donor and recipient, uterine quality (tone, edema, echotexture, and morpho-echogenicity) and ovarian quality (presence of CL and CL size and echogenicity). The effect of reproductive history was not included in the present analysis, as mares with reproductive failures (those that received more than three embryos and did not become pregnant) were excluded from the experiment. It is known that the degree of ovulation synchrony between the donor and recipient may affect P/ET. Recipients in the present study were between 3 and 9 days after ovulation, but P/ET was not influenced by the day of ovulation. This result is in agreement with previous reports (JACOB et al., 2012; RUA et al., 2018), which indicated that a larger synchrony window can be performed in horses compared to cattle (WILSHER et al., 2006). This large window of recipient use allows opportunities for selection during several days per estrous cycle in a large commercial ET program where embryos are collected daily.

Recipients with high endometrial edema and reduced tone have a greater embryonic loss and lower P/ET, which may indicate a uterine environment incompatible with embryonic development (SQUIRES et al., 1999; CARNEVALE et al., 2000; SQUIRES et al., 2003). In this regard, Fleury et al. (2006) suggested that morpho-echogenicity and tone of the uterus have to take into account when choosing the recipients in commercial ET programs. In our study, none of the uterine characteristics assessed by palpation and B-mode ultrasonography significantly affected P/ET or differed between pregnant and non-pregnant recipients, which may reflect the homogeneity of recipients previously selected based on these endpoints.

Regarding the CL-related characteristics in recipients, there is no consensus in previous reports on the effects of CL size and blood perfusion and P4 concentration on pregnancy success after ET in horses. An effect of CL size or CL echogenicity on gestational success was not detected in the present study, suggesting that these morphological endpoints are not accurate for identifying the recipients most likely to maintain pregnancy, corroborating with Arruda et al. (2001). On the other hand, luteal blood perfusion was greater and plasma P4 concentrations tended to be greater in pregnant animals. Previous attempts did not detect a significant difference in CL blood perfusion at the time of ET between pregnant and non-pregnant equine recipients (BROGAN et al., 2016; SALES et al., 2021). This inconsistency between the results observed here and in the earlier reports can be explained by the restricted number of animals (33 to 48 recipients). Unlike other works that used color pixel counting, which cannot be performed in real-time. In our study, we subjectively evaluated luteal blood perfusion in real-time (highly efficient) and classified the animals into two groups (high or low luteal blood perfusion), simplifying and optimizing the field evaluation, which contributed to the innovative and positive results reported here.

For a powerful analysis of factors that can affect a binomial variable such as P/ET, a large number of replicates has to be used to reduce the risk of conclusions based on type II error. In the present study, we were able to determine the impact of luteal blood perfusion in 110 recipients at the time of ET using a practical and real-time approach to examine by Doppler ultrasonography. Therefore, our first hypothesis that CL blood perfusion has a greater impact than CL size on P/ET after ET was tested in a large population and was fully supported. First, there was no significant effect of CL size on P/ET, whereas a considerable 35.8% increase in P/ET was observed in animals with high luteal blood perfusion. Also, in the ROC curve analysis, only luteal blood perfusion was detected as a significant predictor of pregnancy in mares at the time of ET.

Furthermore, our findings indicated that a greater CL activity indicated by CL blood perfusion is positively associated with pregnancy rates. When luteal blood perfusion was evaluated as a continuous variable, a positive and linear association between luteal blood perfusion and the probability of pregnancy was observed. A cubic effect was also detected, indicating a negative relationship between the probability of pregnancy and luteal blood perfusion until the CL reaches 45% perfusion, followed by a positive relationship up to 75% and a further negative relationship up to 85%. This possible negative relationship between P/ET and luteal blood perfusion in recipients with luteal perfusion < 45% and > 75% has to be taken carefully as a restricted number of animals presented values up to 45% (n= 19) or > 75% (n= 7). Also, the age of the recipients with CL blood perfusion of up to 45% may have influenced this cubic relationship. Coincidentally, most animals with luteal perfusion <45% were young animals (13 out of 19; aged between 3 and 9 years old), including six virgin fillies, which may have contributed to the increased P/ET and consequently this initial negative relationship. In this regard, several studies have reported that young animals, mainly virgin fillies, have higher P/ET compared to older animals (SCOGGIN, 2015; HANLON et al., 2012; NATH et al., 2010; HOLLINSHEAD et al., 2022).

The mechanisms involved in increasing pregnancy maintenance in recipient mares with high blood perfusion of CL are not known but apparently are not associated with a blood supply-induced increase in P4 synthesis. In the present study, P4 concentrations were similar between mares with high and low blood perfusion. In addition, no significant correlation was observed between the blood perfusion and plasma P4.

Alternatively, CL blood perfusion may be associated with the expression of factors related to angiogenesis, which are critical to the development and maintenance of CL function (TAMURA & GREENWALD, 1987; FRASER et al., 2000; MATSUOKA-SAKATA et al., 2006). Angiogenesis is composed of condensed blood vasculature and develops under the influence of angiogenic factors, stimulated by vascular endothelial growth factor (VEGF) and basic fibroblastic growth factor, among others (REYNOLDS & REDMER, 1999; BERISHA & SCHAMS, 2005). The VEGF is a potent angiogenic and survival factor for endothelial cells during angiogenesis (FERRARA & DAVIS-SMYTH, 1997; FERRARA et al., 2003; AL-ZIAB et al., 2003). Angiopoietins are another important vascular growth factor that acts in conjunction with VEGF for the formation, stabilization, and regression of blood vessels during divergent phases of CL formation, regression, and rescue by pregnancy (SURI et al., 1996; THURSTON et al., 2000; SUGINO et al., 2005). A high stabilization of blood vessels in the CL is a prerequisite factor for the rescue of CL at the beginning of maternal recognition and pregnancy (SUGINO et al., 2005). Therefore, the presence of a CL with greater CL blood perfusion in the present study could be associated with a greater luteal expression of VEGF and angiopoietins, which could be improving the resistance of the CL to luteolytic factors and favor pregnancy maintenance. However, more investigations are necessary to elucidate the role of luteal blood perfusion, angiogenesis, and vasoactive factors in CL function and pregnancy maintenance in mares.

In addition to CL blood perfusion, plasma P4 concentrations also affected P/ET in the current study. There are not many studies evaluating the impact of P4 on P/ET in horses and the reports presented ambiguous results. Souza (2006) suggested that recipients with a higher concentration of P4 are more able to maintain the pregnancy, but Hollinshead et al. (2022) did not observe an effect of P4 on P/ET in cyclic recipient mares. Other reports (KNOWLES et al., 1993; ALONSO, 2007) suggest that the circulating P4 concentrations are not associated with the P/ET, but a minimum concentration is required to guarantee pregnancy maintenance. In studies carried out in ovariectomized mares supplemented with P4 after ET, a P4 concentration < 4 ng/mL was associated with a lower P/ET, and a concentration ≥ 4 ng/mL was necessary to maintain pregnancy in these recipients (SHIDELER et al., 1982; GINTHER et al., 1985; KNOWLES et al., 1993; HOLLINSHEAD et al., 2022). Several studies in mares suggested that there is a close relationship between luteal blood flow and luteal P4 synthesis and that a high blood supply to the CL represents an important requirement for P4 production (BOLLWEIN et al., 2002a; GINTHER et al., 2006; GINTHER et al., 2007). Although these reports suggested that CL blood perfusion and P4 synthesis are highly associated in several species, it is likely that the effects of both traits on P/ET in the present study are synergic rather than correlated. In addition to the absence of a significant correlation between luteal blood perfusion and P4 at the time of ET, when P/ET was studied according to these two endpoints, the highest P/ET was obtained in recipients with high luteal blood perfusion and high P4, and the lowest in those with low blood perfusion and low P4.

Thus, the present results suggest that luteal blood perfusion and circulating P4 before ET are interesting pregnancy success predictors, but unlike the blood perfusion assessment of the CL through Doppler ultrasonography, the use of P4 is limited as it cannot be performed in real-time. The commercial assays for circulating P4 is time-consuming and with limited use under field conditions. Alternatively, real-time assessment of luteal blood perfusion using color-Doppler imaging allows the selection of highly receptive mares, which can considerably improve the pregnancy rate in equine ET programs if recipients with high luteal blood perfusion are selected. Therefore, Doppler imaging could reduce the proportion of recipients susceptible to failures in establishing pregnancy in ET programs in horses, since the embryos would not be transferred to recipients with sub-functional CL, as already reported in cattle (PINAFFI et al., 2015; PUGLIESI et al., 2019). Furthermore, when there is an excessive number of recipients considered able to receive an embryo after the conventional pre-selection of synchronized animals, those with luteal perfusion ≥ 55% can be prioritized. So, this strategy could be used as an alternative to improve the chances of establishing pregnancy of high genetic merit and value embryos in commercial ET programs.

5 CONCLUSIONS

We conclude that in pre-selected recipient mares, the CL size or uterine-accessed characteristics determined by transrectal palpation or by B- and color-Doppler ultrasonography do not influence P/ET. Luteal blood perfusion determined by color-Doppler ultrasonography was the only reproductive characteristic at the ET that differed between pregnant and non-pregnant recipients. Luteal blood perfusion and plasma P4 at the time of ET are not correlated but both factors independently affected the P/ET and these effects are likely synergic to improve pregnancy maintenance. Finally, luteal blood perfusion was identified as the most efficient endpoint for pregnancy predictor and can be used as an additional real-time evaluation for selection of recipient mares to improve pregnancy success in commercial ET programs.

6 FINAL CONSIDERATIONS

Although P4 concentration is a potential indicator of gestational success, it cannot be used in real-time to select the most suitable recipient in a commercial ET program. In addition, luteal blood perfusion determined by Doppler ultrasonography is an innovative and easily accessible tool that can be used in real-time and with high repeatability to select recipients with a greater chance of maintaining pregnancy in ET programs in horses, based on the luteal blood perfusion. The use of Doppler ultrasonography for selection of recipients is still restricted but it is already commercially used in ET programs in cattle. Although its use in horses has been tested in a few previous research reports, the lack of a positive impact on ET success has limited its use in commercial programs in horses. Thus, the herein pioneer results showing a positive association between CL blood perfusion and pregnancy success, open new possibilities of investigations to support the effectiveness of this technique for selection of recipients in commercial ET programs in horses.

These results are encouraging and encourage veterinarians to carry out the selection of equine recipients through analysis of luteal blood perfusion performed by Doppler ultrasonography. This technique allows the selection of the recipient with the greatest potential to maintain pregnancy and increase success in commercial ET programs in horses. It is important to emphasize that Doppler ultrasonography for the selection of equine recipients does not replace the conventional evaluation (palpation + B-mode), but it comes to add to the decision-making process and potentially improves pregnancy rates after ET.

Considerable progress has already been made regarding molecular events that are associated with CL angiogenesis, especially in rats, primates, and humans, but studies in large animals, especially in horses, are still scarce. The lack of clarity on the changes in vascular CL perfusion and the pregnancy events may be partly due to the different results among species.

Faced with the scarcity of studies correlating the high vascularization of CL with the physiological mechanisms that may be involved in the success of pregnancy, there is a wide range to study the physiological processes of angiogenesis in high perfusion CL and its relationship with the maintenance of pregnancy in mares. After all, for the maintenance of pregnancy to occur, the life of the CL needs to be extended. One of the questions to be answered is whether pregnancy maintenance involves greater angiogenesis and greater vessel stability and the CL failure and early gestational loss are due to reduced CL vascularization.

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