Power Doppler can detect the presence of 7–8 day conceptuses prior to flushing in an equine embryo transfer program

Published Version:
Power Doppler can detect the presence of 7–8 day conceptuses prior to flushing in an equine embryo transfer program / Nieto-Olmedo P.; Martin-Cano F.E.; Gaitskell-Phillips G.; Ortiz-Rodriguez J.M.; Pena F.J.; Ortega-Ferrusola C.. - In: THERIOGENOLOGY. - ISSN 0093-691X. - ELETTRONICO. - 145:(2020), pp. 1-13. [10.1016/j.theriogenology.2020.01.015]

Availability:
This version is available at: https://hdl.handle.net/11585/927674 since: 2023-06-09

Published:
DOI: http://doi.org/10.1016/j.theriogenology.2020.01.015

Power Doppler can detect the presence of 7-8 day conceptuses prior to flushing in an equine embryo transfer program

P. Nieto-Olmedo, DVM [a], F.E. Martín-Cano, DVM, PhD [b], G. Gaitskell-Phillips, DVM [b], J.M. Ortiz-Rodríguez, DVM [b], F.J. Pe~na, DVM, PhD [b], C. Ortega-Ferrusola, DVM, PhD [b, *]

[a] CEFIVA-Centro de Fertilizaci_on In vitro de Asturias, Spain
[b] Laboratory of Equine Reproduction and Equine Spermatology, Veterinary Teaching Hospital, University of Extremadura, C_aceres, Spain

Abstract

In order to determine whether differences in uterine blood flow between pregnant and non-pregnant mares can be used to predict the presence of the equine embryo prior to flushing in an embryo transfer program, power Doppler ultrasonography was used on a total of 52 mares on days 7 or 8 postovulation. Computer analysis of Doppler images was subsequently performed using ImageJ v1.48 software. Vascular perfusion of the endometrium was analyzed using spot meter techniques, measuring mean pixel intensity and area of blood flow. Mares with positive flushings presented a higher uterine blood flow area (one embryo: 54.01 ± 2.27 mm2 or two embryos: 61.01 ± 6.73 mm2) prior to embryo recovery compared to barren mares (21.77 ± 2.22 mm2) ($p \leq 0.05$). However, significant differences in vascular perfusion were not detected between single or twin pregnancies. Blood flow area appears to be a good predictor for differentiation between pregnant and non-pregnant mares with an AUC: 0.869; $p \leq 0.001$ and an optimal cut-off value of 37.21 mm2. Both the mare's age and day of embryo recovery caused effects on uterine vascular perfusion. According to Youden's J statistics the uterine blood flow area of young pregnant mares was greater than 25.4 mm2 on day 7 (with a sensitivity of 75% and a specificity of 87.5%) and greater than 21.02 mm2 on day 8 post-ovulation (with a sensitivity of 93.8% and a specificity of 100%). The uterine blood flow area in adult pregnant mares was greater than 41.4 mm2 on day 7 (with a sensitivity of 80% and a specificity of 85.5%) and greater than 35.55 mm2 on day 8 after ovulation (with a sensitivity of 97.2% and a specificity of 85.7%). Evaluation on day 8 is therefore considered to be more reliable. Older and middle aged pregnant mares (5e18 years old) had increased uterine vascularization compared to young pregnant mares (2e5 years old) (p 0.001). Conversely, older barren mares showed higher endometrial vascularity (35.06 ± 2.56 mm2) than young (17.21 ± 1.26 mm2) and middle aged nonpregnant mares (23.84 ± 1.50 mm2) (p 0.05). We hypothesized that the higher blood flow area seen in older barren mares may be a consequence of a subclinical endometritis due to repeated flushing for embryo recovery. The results of the present study indicate that power Doppler ultrasound combined with computer assisted analysis of images are reliable techniques to detect early pregnancy prior to embryo recovery.

Keywords: Embryo development, Uterine blood flow, Endometrial angiogenesis, Maternal recognition of pregnancy, Doppler ultrasonography, Mare

1. Introduction

Embryo transfer (ET) is a well-established assisted reproductive technique in the equine industry. Despite the fact that in mares the fertilization rate is greater than 90%, the expected embryo recovery rate is only about 75% [1e4]. Flushing for embryo recovery from the donor mare's uterus is normally performed on day 7 or 8 postovulation, without a previous pregnancy diagnosis. The equine presents a high incidence of early pregnancy loss, with losses between 5 and 15% in young fertile mares which reach higher values in older mares, especially after the use of biotechnologies [5,6]. Moreover, these percentages of pregnancy loss, could possibly be even higher, since the presence of the embryo cannot be detected before day 11e12 post-ovulation using conventional ultrasonography [2,7].

Equine embryos enter the uterine lumen between 5.5 and 6 days after ovulation, and the intrauterine mobility phase commences and continues until fixation occurs (day 16e17) [8,9].
Previous research has revealed that the migration of the embryo could also have a local effect on endometrial vascularization, stimulating uterine blood flow [10]. Moreover, this increase in vascular perfusion during early pregnancy has been detected in ruminants and sows, and is restricted to the uterine area that is in direct contact with the conceptus [11e13]. Doppler ultrasound is widely used for the early diagnosis of subfertility problems in human [14] and equine reproduction [15e17], as well as being a useful tool to assess the vascular perfusion of the genital tract after surgical or medical treatments [18e20]. Color-Doppler sonography has become one of the most reliable non-invasive methods for assessment of the functionality of the CL and uterine vascular hemodynamics in large animals [21e23]. In addition, this diagnostic tool has previously been used in embryo transfer programs to assess CL function in recipients mares [24] and cows [25]. However, to the best of our knowledge, assessment of uterine vascular perfusion as an indicator of early pregnancy prior to embryo recovery, has not yet been investigated. Earlier studies using color and pulse Doppler ultrasound evaluated the blood flow of uterine arteries during early pregnancy in mares [10,26]. However, differences in vascular perfusion between pregnant and non-pregnant mares were not detected until day 11 post ovulation, probably due to the lower sensitivity of color-mode Doppler for detection of vessels with small diameters. Power-mode Doppler could be a more suitable ultrasonographic modality to measure early variations in blood flow. Recently, computer assisted analysis of ultrasonographic images using specific software is contributing towards providing objective measures of blood flow [26,27]. Therefore, we hypothesize that power Doppler ultrasound could be a good predictor of early pregnancy in an embryo transfer program. The goal of this studywas to investigate whether uterine blood flowcould be a good indicator of the presence of embryo as early as 7- or 8-days post-ovulation, before uterine flushing in an embryo transfer program.

2. Materials and methods

2.1. Experimental design

In order to determine whether power-mode Doppler ultrasonography is able to detect differences in uterine blood flow between pregnant and non-pregnant mares, a total of 52 mares were classified into two groups: Group 1: 26 mares where embryo recovery was performed on day 7; and group 2: 26 mares where embryo recovery was performed on day 8. From 52 mares, 44 of the mares were inseminated with stallions of proven fertility close to ovulation and the other 8 formed the negative control group (noninseminated mares). Doppler assessment of uterine blood flow was performed before embryo recovery on day 7 or 8 after ovulation. The number of embryos and pregnancy diagnosis after the embryo transfer were recorded. Doppler analysis was subsequently performed blinded, off-line on a computer.

2.2. Animals

Animals belonging to and housed in our institution were kept according to institutional and European animal care regulations (Law 6/2913 June 11 and European Directive 2010/63/EU), and all experimental procedures were reviewed and approved by the Ethical committee of the University of Extremadura, Caceres, Spain. A total of 52 mares (Pure Bred Spanish horses) between 2 and 18 years old were used in this study. All mares were evaluated prior to the study (one cycle) by ultrasonographic examination and tested by culture of swabs. Mares with uterine pathological signs such as endometrial cysts, uterine fluid accumulation, irregular cycles and hyperedema grade 5/5 were excluded from the study. One cycle per mare was studied. From 52 mares in this study, 44 were inseminated with fresh semen and induced to ovulate on the same day and 8 were used as controls (not inseminated).

2.3. Artificial insemination

All mares were scanned daily until a preovulatory follicle of at least 35mmand a uterine edema grade 3/5 were detected. Then, all mares were induced to ovulate by administration of 2500 IU of hCG (Veterin Corion®) IV and inseminated 24 h after induction with a standard dose of fresh semen from different stallions (20 ml, 500 _ 106 mill spz with progressive motility). Mares were checked ultrasonographically every 12 h until ovulation. Day of ovulation was considered as day 0.

2.4. Ultrasonographic examination and image analysis

Evaluation of uterine vascular hemodynamics and subsequent uterine flushing for embryo recovery were performed on day 7 (Group 1: 22 mares and 4 control mares) and on day 8 (Group 2: 22 mares and 4 control mares) post ovulation. All mares were sedated with romifidine (0.02 mg/kg) (Sedivet® Boehringer Ingelheim, Barcelona, Espa~na) before commencing ultrasonographic assessments. Ultrasonographic examination was performed using MyLabFive Vet equipment (Esaote S.p.A, Genova, Italy) and a linear 5e7.5 MHz probe. The settings were: gain: 70%, PRF: 1,4 KHz with a depth of 9 cm. The probe was placed transversely in the middle of each uterine horn and two frozen images were taken per horn. Power flow modes were used to display signals for blood flowin the vessels of all the endometrium, myometrium and perimetrium due to higher sensitivity for detection of weak signals from small vessels. The degree of uterine vascular perfusion was objectively assessed by quantification of pixels in a bitmap (BMP) format. A total of 208 images (four images per mare) were analyzed blindly. Computer analysis of Doppler images was performed at a later date using ImageJ v1.48 software (National Institute of Health, USA). ImageJ is a Java-based image processing program that can calculate area and pixel value statistics of user-defined selections and intensity-thresholded objects. Vascular perfusion of the endometrium was analyzed using spot meter techniques, measuring the mean pixel intensity and area of blood flow. First, blood vessels were detected in the images by applying a color threshold restricted to the uterine horn. After this, mean pixel intensity and total area of the measurement were calculated (Fig. 1). The resultsof the area (pixels) were transformed to mm2 and the mean pixel intensity was expressed in arbitrary units (AU). To do this, the number of pixels in a given distance are quantified and the number of pixels in this distance are normalized.

2.5. Flushing recovery

Non-surgical embryo collection by uterine flushing was performed after Doppler evaluation (day 7 or 8). Mares were flushed to collect embryos three times with 1000 ml of flushing media (Biolife, Agtech, USA) per infusion, using a silicone 32 French catheter (Foley Agtech, USA). The effluent was collected through Y-junction tubing (Agtech, USA) into a 75-microm filter (Agtech, USA). When searching for the embryo, the content of the filter was rinsed into collection dishes which were searched using a stereomicroscope at 20X for the presence of an embryo. Then embryos which were collected were transferred to a recipient mare. The number of embryos collected and positive pregnancy diagnoses were recorded.

2.6. Statistical analysis

Statistical analysis was carried out using the SPSS software package (version 21.0 for Mac), and differences were considered statistically significant with a $p \leq 0.05$. The Shapiro-Wilk test was used to test for normal distribution of the data. The data concerning area presented a normal distribution, and the effect of the number of embryos, the day of embryo recovery and the age of mare were tested using a one-way ANOVA. These data were subjected to Tuckey's tests to determine significance. A Kruskal-Wallis test was used for the pixel intensity data (not normally distributed). All data were presented as mean ± SEM.

Receiver operating characteristic (ROC) curves and Youden's J statistics were used to investigate the value of the proposed variables as indicators of early pregnancy and cut-off values were also established. Receiver operating characteristics (ROC) analyses were used expressing prognostic value as area under curve (AUC) with a 95% confidence interval (CI) and significance test. Results were expressed as mean ± SEM.

3. Results

3.1. Embryo recovery rate

The percentage of positive flushings was 75% (11 negative flushings out of 44) with a total of 40 embryos recovered from 44 flushing operations (26 flushings where one embryo was recovered and 7 flushings where two embryos were recovered) (Table 1).

3.2. Uterine blood flow area in pregnant and non-pregnant mares

In this study, we assessed the area of the blood flow in the uterus of control mares (non-inseminated mares), mares with a negative flushing, mares with one recovered embryo and mares with two recovered embryos. There were no significant differences between control mares (27.43 ± 1.31 mm2) and barren mares (21.77 ± 2.22 mm2) in uterine blood flow area (Fig. 2).

In contrast, mares with positive flushings presented an increase in the area of uterine vascular blood flow (one embryo: 54.01 ± 2.27 or two embryos: 61.01 ± 6.73 mm2) compared with control or barren mares ($p \leq 0.05$) (Figs. 2 and 3). However, regarding the number of embryos, there were no differences between the presence of one or two embryos (Fig. 2).

Similarly, the intensity of blood flow was evaluated, but there were no differences between groups (Fig. 4).

3.3. Effect of the day of embryo recovery on uterine blood flow

The area of uterine blood flow in pregnant mares was significantly higher on day 8 than on day 7 (63.12 ± 2.61 vs 47.40 ± 2.69; $p \leq 0.05$). However, no increase in the blood flow in non-pregnant mares was detected (Fig. 5).

3.4. Effect of mare age on the uterine blood flow

Mares were classified based on their age into three groups: Young mares (2-5 years old), adult middle aged mares (6-10 years old) and old mares (>11 years old). The age of the mare caused effects on uterine vascular perfusion. Older and middle aged pregnant mares (adult mares) had higher uterine blood flow than young pregnant mares (p ≤ 0.05). However, there were no differences in uterine blood flow between young and middle aged nonpregnant mares (Table 3).

3.5. Forecasting the presence of the embryo using ROC curves

The area of uterine blood flow was further investigated using ROC curves and Youden's J index statistics. Differentiation between pregnant and non-pregnant mares (AUC: 0.869; p 0,001) was possible with this technique. The optimal cut-off value was 37.21 mm2. In general, non-pregnant mares reliably fell below this value of cut-off (Sensitivity: 76.5 and Specificity 89.5) (Fig. 6).
The cut-off values for the area of blood flow were also established taking into account the age of the mare and the day of pregnancy (Fig. 7). Results are shown in the table (Table 4).

4. Discussion

Recent advances in reproductive biotechnology and medicine have improved pregnancy outcome in equines. In spite of this, the incidence of early pregnancy death continues to be one of the main sources of economic loss to the equine breeding industry. Pregnancy loss rates in mares are from 5 to 15% in young fertile mares, but may increase to values higher than 20% in older mares, particularly after the use of biotechnologies such as embryo transfer or insemination with frozen-thawed semen [5,6]. Moreover, these percentages of pregnancy loss may possibly be even higher, since we cannot detect the presence of the embryo before day 11e12 post-ovulation. To date, the availability of techniques for detection of early pregnancy loss in mares have been limited. The results of the present study indicate, for the first time, that power Doppler ultrasound combined with computer assisted analysis of images are reliable and effective techniques for the detection of early pregnancy before day 10 in mares. To the best of our knowledge, this is the first report where differences in uterine vascular perfusion between pregnant and non-pregnant mares as early as 7- and 8-days post-ovulation have been detected. Previous research also assessed uterine vascularization using color and spectral Doppler mode. However, significant differences between pregnant and barren mares were not seen until day 11 post-ovulation [26]. Bollwein et al. (2003) observed a gradual increase in blood flow in both uterine arteries during the embryo's mobile phase [10], unlike in other species, where greater vascular perfusion was only detected in the ipsilateral uterine artery to where the embryo was implanted. We did not find differences in blood flow area between uterine horns (data not shown), but a general increase in the vascularization in pregnant mares. Studies in sows and cows attributed the increase in the blood flow to synthesis and release of substances with vasoactive functions such as estradiol -17β by the embryo [27e29]. Estrogen increases uterine blood flow by binding to receptors in the uterine artery wall, and in particular the uterine artery endothelium [30]. Circulating estrogens have a vasodilator effect on the uterine and ovarian arteries in cycling mares and after superovulation treatments [31,32]. The equine embryo also synthetizes estrogens from day 6 post ovulation onwards, with a higher production as the embryo growths [33]. In our study, pregnant mares showed a significant increase in uterine blood flow area on day 7 post ovulation, which was higher on day 8. In contrast, in the control and non-pregnant groups, mean uterine blood flow area was lower than in pregnant mares and this parameter did not change on any of the days studied. Moreover, we did not find significant differences in uterine hemodynamics between single or twin pregnancies, although we observed a slightly higher uterine perfusion with twins (p: 0.06).
Conversely, recent studies demonstrated that equine embryos produce other vasoactive substances such a PGE and PGF2a that may also stimulate uterine blood flow. These substances in addition to vascular endothelial growth factors (VEGF) synthetized by the endometrium of the mare are involved in the regulation of endometrial angiogenesis [8,34,35]. Moreover, the mobility of the embryo

through the uterus might distribute these substances throughout the entire endometrium. During early pregnancy in the mare the endometrial secretion of prostaglandin F2a is temporarily reduced [36]. Similarly, responsiveness to oxytocin is also altered due to reduced expression of the oxytocin receptor [37]. Therefore, embryonic mobility is most likely induced by prostaglandins synthesized and secreted from the conceptus itself. The embryo enters the uterine cavity [8,34,38], also stimulating uterine blood flow. Thus, the increase in uterine blood flow in pregnant mares during early pregnancy may be related to the vasodilator effect caused by estradiol and other vasoactive substances such as VEGF secreted by the embryo.

Additionally, this higher blood flow detected in pregnant mares could also be due to conceptus-derived relaxin. A recent transcriptomic study revealed that the equine embryo expresses the relaxin transcript during the mobile phase with levels increasing as the conceptus develops [39,40]. In horses, the embryo and the corpus luteus appear to be the only sources of relaxin, since relaxin transcripts were not detected in the equine endometrium during this period of development [40]. Relaxin belongs to the insulin superfamily and its potential angiogenic effect on different tissues is well defined [41,42]. During early pregnancy, relaxin stimulates endometrial blood flow through upregulation of endometrial expression of vascular endothelial growth factor (VEGF) [43]. In fact, a recent report demonstrated that pregnant mares showed a higher expression of VEGF and VEGFR2 mRNA when they were compared with cyclic mares [35]. Recently, a study in bovines showed that their embryos also express transcripts for genes associated with angiogenesis (VEGF) [44]. All these findings suggest that the conceptus plays an active role in endometrial vascular remodeling within the uterus required for its fixation and implantation.

One of the indications for embryo transfer is for recovery of embryos from donor mares that are capable of getting pregnant but which undergo repeated early pregnancy loss associated with factors such as uterine periglandular fibrosis or other age-related uterine changes [45]. Regarding this, we classified mares into three groups, aiming to evaluate whether uterine vascular perfusion may be affected by age. Several studies have suggested that age increases the incidence of uterine angiopathies in mares [46,47] and could have important implications in endometrial glandular development, post-breeding endometritis and early pregnancy death in aged mares [48,49]. Moreover, in a previous study, transrectal color and power Doppler ultrasonography was used to study uterine vascular perfusion in mares with and without uterine cysts. Reduced uterine vascular perfusion was detected in mares with uterine cysts [50]. In contrast to these studies, we detected increased endometrial blood flow area in older and adult pregnant mares than in young pregnant mares ($p \leq 0.001$) although there was no evidence of endometrial cysts in the mares included in this study. Conversely, older non-pregnant mares showed a higher endometrial vascularity (35.06 ± 2.56 mm2) when they were compared with young (17.21 ± 1.26 mm2) and middle aged nonpregnant mares (23.84 ± 1.50 mm2) (p 0.05). These differences in blood flow area, may originate due to a subclinical endometritis in older mares, as a result of repeated flushing for embryo recovery. In this study we did not perform histopathological evaluation of the endometrium of these mares but previous reports demonstrated a positive correlation between repeated embryo recovery attempts and chronic inflammatory changes in the uterus [51]. Moreover, there is clinical evidence indicating that repeated flushing-induced endometritis in mares and recurring acute endometritis may result in chronic degenerative fibrotic changes to the endometrium [52,53].

Doppler ultrasonography is a non-invasive technique that provides qualitative information about blood flow to different organs. The results of this article have a clinical relevance in routine practice. Data from the present study reveals that mares with positive flushings present an increase in uterine blood flow area (one embryo: 54.01 ± 2.27 mm2 or two embryos: 61.01 ± 6.73 mm2) compared with barren mares (21.77 ± 2.22 mm2) ($p \leq 0.05$). Accuracy of this parameter for differentiation of pregnant from nonpregnant mares was evaluated by the area under the ROC curve (AUC). Area of

blood flow (ABF) presented AUC values of 0.869 ($p \leq 0.001$). According to this statistical method, this parameter is considered to be a “good indicator” for the early diagnosis of pregnant mares. The optimal cut-off value of ABF to diagnose pregnant mares was 37.21 mm2. In general, non-pregnant mares reliably fell below this cut-off value (Sensitivity: 76.5 and Specificity 89.5). Because of the effect of the age of the mare and the effect of the day of embryo transfer on vascular perfusion of the uterus, we also established cut-off values for blood flowarea for day 7 and 8 in young and adult mares. The most accurate date to diagnose the presence of the embryo in the uterus was day 8 postovulation, with an AUC of 0.97 in young mares and 0.94 in adult mares. Therefore, we suggest that the best day to predict if the mare is pregnant or not is day 8 post-ovulation and the cut off values for young pregnant mares (2-5 years) is 21.02mm and for adult mares (>6 years) is 41.36 mm. The cut off values obtained in this study can be used as a reference with different ultrasound equipment providing that the settings detailed in the study are used.
Previous studies in human reproduction demonstrated that preconceptional uterine blood flow, as well as the expression of endometrial genes is involved in endometrial receptivity and implantation in women [54,55]. Moreover, endometrial parameters of blood flow were good predictors of pregnancy outcome after embryo transfers [56]. Uterine receptivity for implantation is crucial for survival of the embryo, as well as embryo signaling during maternal recognition of pregnancy. Deficiencies in endometrial receptivity and/or anomalies in the embryo signaling processes could be identified as early lower uterine area of blood flow. These results support the need for further research into uterine vascular perfusion as a potential marker of early pregnancy loss or even placental dysfunction. This technique can be applied in practice by an experienced ultrasound operator, using the recommended settings and cut-off values. The possibility of diagnosing an early pregnancy based on endometrial blood flow represents a significant advance in reproductive medicine and biotechnologies such as embryo transfer. However, having to analyze images using external software is an inconvenience of this technique, since means that immediate results cannot be obtained. However, the complete analysis of the images does not take more than 15 min. The inclusion of this kind of software within existing ultrasound devices for application in reproductive medicine would be very advantageous for clinicians and researchers. Power-Doppler ultrasound may also became a potential tool for the diagnosis of early pregnancy loss in mares, although further research in the area is needed.

5. Conclusion

In conclusion, the results of the present study indicate that power Doppler ultrasound combined with computer assisted analysis of images are reliable techniques for detection of early pregnancy prior to embryo recovery on day 7- and 8 postovulation. The technique could be used in routine clinical practice as a way of maximizing embryo recovery rates from donor mares. Cut-off values established in this study can be used to predict pregnancy diagnosis prior to embryo collection.

Funding
This work was supported by the Ministerio de Economía y Competitividad-FEDER (GR AGL2017-83149-R) and Junta de Extremadura-FEDER (GR 18008 and IB16030). JMOR holds a PhD grant from the Junta de Extremadura- FEDER (PD 18005).

Author contribution section
PNO: Conceptualization, conceived the study and performed experiments, methodology and data curation; FEMC: performed the analysis of data with Image J (Software), validation and reviewed the draft. GGF: English review (native speaker) Writing - review & editing, Software. JMO: Methodology,

data curation. FJP: Conceptualization, funding acquisition, English review and statistical analysis, COF: Conceptualization, conceived the study, supervision, formal analysis and roles/Writing - original draft.

Declaration of competing interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Acknowledgements
The authors want to thank to “Esaote Spain”, in particular Juan Carlos, Afrodita and the Borja family for their constant collaboration with us, providing us with the ultrasound equipment to perform this study.

References
[1] Aurich C, Budik S. Early pregnancy in the horse revisited - does exception prove the rule? J Anim Sci Biotechnol 2015;6:50.
[2] Ginther OJ, Garcia MC, Bergfelt DR, Leith GS, Scraba ST. Embryonic loss in mares: pregnancy rate, length of interovulatory intervals, and progesterone concentrations associated with loss during days 11 to 15. Theriogenology 1985;24:409e17.
[3] Weber JA, Freeman DA, Vanderwall DK, Woods GL. Prostaglandin E2 secretion by oviductal transport-stage equine embryos. Biol Reprod 1991;45:540e3.
[4] Squires EL, McCue PM, Vanderwall D. The current status of equine embryo transfer. Theriogenology 1999;51:91e104.
[5] Carnevale EM, Ramirez RJ, Squires EL, Alvarenga MA, Vanderwall DK, McCue PM. Factors affecting pregnancy rates and early embryonic death after equine embryo transfer. Theriogenology 2000;54:965e79.
[6] Ortiz-Rodriguez JM, Ortega-Ferrusola C, Gil MC, Martin-Cano FE, Gaitskell-Phillips G, Rodriguez-Martinez H, et al. Transcriptome analysis reveals that fertilization with cryopreserved sperm downregulates genes relevant for early embryo development in the horse. PLoS One 2019;14:e0213420.
[7] Ginther OJ, Bergfelt DR, Leith GS, Scraba ST. Embryonic loss in mares: incidence and ultrasonic morphology. Theriogenology 1985;24:73e86.
[8] Stout TA, Allen WR. Role of prostaglandins in intrauterine migration of the equine conceptus. Reproduction 2001;121:771e5.
[9] Ginther OJ. Mobility of the early equine conceptus. Theriogenology 1983;19:603e11.
[10] Bollwein H, Mayer R, Stolla R. Transrectal Doppler sonography of uterine blood flow during early pregnancy in mares. Theriogenology 2003;60: 597e605.
[11] Ford SP, Chenault JR, Echternkamp SE. Uterine blood flow of cows during the oestrous cycle and early pregnancy: effect of the conceptus on the uterine blood supply. J Reprod Fertil 1979;56:53e62.
[12] Ford SP, Christenson RK. Blood flow to uteri of sows during the estrous cycle and early pregnancy: local effect of the conceptus on the uterine blood supply. Biol Reprod 1979;21:617e24.
[13] Greiss Jr FC, Anderson SG. Uterine blood flow during early ovine pregnancy. Am J Obstet Gynecol 1970;106:30e8.
[14] El-Mazny A, Abou-Salem N, Elshenoufy H. Doppler study of uterine hemodynamics in women with unexplained infertility. Eur J Obstet Gynecol Reprod Biol 2013;171:84e7.
[15] Ortega-Ferrusola C, Gracia-Calvo LA, Ezquerra J, Pena FJ. Use of colour and spectral Doppler ultrasonography in stallion andrology. Reprod Domest Anim 2014;49(Suppl 4):88e96.
[16] Ortiz-Rodriguez JM, Anel-Lopez L, Martin-Munoz P, Alvarez M, Gaitskell-Phillips G, Anel L, et al. Pulse Doppler ultrasound as a tool for the diagnosis of chronic testicular dysfunction in stallions. PLoS One 2017;12. e0175878.
[17] Ginther OJMD. Doppler ultrasound in equine reproduction: principles, techniques, and potential. J Equine Vet Sci 2004;24:516e26.

[18] Pozor MA, Muehlhaus J, King A, Macpherson ML, Troedsson MH, Bailey CS. Effect of pentoxifylline treatment on testicular perfusion and semen quality in Miniature horse stallions. Theriogenology 2011;76:1027e35.
[19] Bailey CS, Sper RB, Schewmaker JL, Buchanan CN, Beachler TM, Pozor MA, et al. Uterine artery blood flow remains unchanged in pregnant mares in response to short-term administration of pentoxifylline. Theriogenology 2012;77:430e6.
[20] Gracia-Calvo LA, Duque J, Balao da Silva C, Ezquerra J, Ortega-Ferrusola C. Testicular perfusion after standing laparoscopic peritoneal flap hernioplasty in stallions. Theriogenology 2015;84:797e804.
[21] Bollwein H, Maierl J, Mayer R, Stolla R. Transrectal color Doppler sonography of the A. uterina in cyclic mares. Theriogenology 1998;49:1483e8.
[22] Bollwein H, Weber F, Woschee I, Stolla R. Transrectal Doppler sonography of uterine and umbilical blood flow during pregnancy in mares. Theriogenology 2004;61:499e509.
[23] Hassan M, Arshad U, Bilal M, Sattar A, Avais M, Bollwein H, et al. Luteal blood flow measured by Doppler ultrasonography during the first three weeks after artificial insemination in pregnant and non-pregnant Bos indicus dairy cows. J Reprod Dev 2019;65:29e36.
[24] Brogan PT, Henning H, Stout TA, de Ruijter-Villani M. Relationship between colour flow Doppler sonographic assessment of corpus luteum activity and progesterone concentrations in mares after embryo transfer. Anim Reprod Sci 2016;166:22e7.
[25] Pugliesi G, Dalmaso de Melo G, Silva JB, Carvalhedo AS, Lopes E, de Siqueira Filho E, et al. Use of color-Doppler ultrasonography for selection of recipients in timed-embryo transfer programs in beef cattle. Theriogenology 2019;135: 73e9.
[26] Silva LA, Gastal EL, Beg MA, Ginther OJ. Changes in vascular perfusion of the endometrium in association with changes in location of the embryonic vesicle in mares. Biol Reprod 2005;72:755e61.
[27] Silva LA, Ginther OJ. An early endometrial vascular indicator of completed orientation of the embryo and the role of dorsal endometrial encroachment in mares. Biol Reprod 2006;74:337e43.
[28] Robertson HA, King GJ. Plasma concentrations of progesterone, oestrone, oestradiol-17beta and of oestrone sulphate in the pig at implantation, during pregnancy and at parturition. J Reprod Fertil 1974;40:133e41.
[29] Ford SP, Christenson RK, Ford JJ. Uterine blood flow and uterine arterial, venous and luminal concentrations of oestrogens on days 11, 13 and 15 after oestrus in pregnant and non-pregnant sows. J Reprod Fertil 1982;64:185e90.
[30] Chenault JR. Steroid metabolism by the early bovine conceptus-I. 5 beta-Reduction of neutral C19-steroids. J Steroid Biochem 1980;13:499e506.
[31] Heryanto B, Rogers PA. Regulation of endometrial endothelial cell proliferation by oestrogen and progesterone in the ovariectomized mouse. Reproduction 2002;123:107e13.
[32] Gastal EL, Gastal MO, Ginther OJ. Relationships of changes in B-mode echotexture and colour-Doppler signals in the wall of the preovulatory follicle to changes in systemic oestradiol concentrations and the effects of human chorionic gonadotrophin in mares. Reproduction 2006;131:699e709.
[33] Witt MC, Bollwein H, Probst J, Baackmann C, Squires EL, Sieme H. Doppler sonography of the uterine and ovarian arteries during a superovulatory program in horses. Theriogenology 2012;77:1406e14.
[34] Heap RB, Hamon M, Allen WR. Studies on oestrogen synthesis by the preimplantation equine conceptus. J Reprod Fertil Suppl 1982;32:343e52.
[35] Stout TA, Allen WR. Prostaglandin E(2) and F(2 alpha) production by equine conceptuses and concentrations in conceptus fluids and uterine flushings recovered from early pregnant and dioestrous mares. Reproduction 2002;123: 261e8.
[36] Silva LA, Klein C, Ealy AD, Sharp DC. Conceptus-mediated endometrial vascular changes during early pregnancy in mares: an anatomic, histomorphometric, and vascular endothelial growth factor receptor system immunolocalization and gene expression study. Reproduction 2011;142: 593e603.
[37] Douglas RH, Ginther OJ. Concentration of prostaglandins F in uterine venous plasma of anesthetized mares during the estrous cycle and early pregnancy. Prostaglandins 1976;11:251e60.
[38] Starbuck GR, Stout TA, Lamming GE, Allen WR, Flint AP. Endometrial oxytocin receptor and uterine prostaglandin secretion in mares during the oestrous cycle and early pregnancy. J Reprod Fertil 1998;113:173e9.

[39] Weber JA, Woods GL, Freeman DA, Vanderwall DK. Prostaglandin E2 secretion by day-6 to day-9 equine embryos. Prostaglandins 1992;43:55e9.
[40] Klein C. The role of relaxin in mare reproductive physiology: a comparative review with other species. Theriogenology 2016;86:451e6.
[41] Klein C, Troedsson MH. Transcriptional profiling of equine conceptuses reveals new aspects of embryo-maternal communication in the horse. Biol Reprod 2011;84:872e85.
[42] Schwabe C, Bullesbach EE. Relaxin: structures, functions, promises, and nonevolution. FASEB J 1994;8:1152e60.
[43] Vandevoort CA, Mtango NR, Latham KE, Stewart DR. Primate preimplantation embryo is a target for relaxin during early pregnancy. Fertil Steril 2011;96: 203e7.
[44] Klein C. Pregnancy recognition and implantation of the conceptus in the mare. Adv Anat Embryol Cell Biol 2015;216:165e88.
[45] Valente RS, Almeida TG, Alves MF, Camargo J, Basso AC, Belaz KRA, et al. Modulation of long-chain Acyl-CoA synthetase on the development, lipid deposit and cryosurvival of in vitro produced bovine embryos. PLoS One 2019;14. e0220731.
[46] Kenney RM. Cyclic and pathologic changes of the mare endometrium as detected by biopsy, with a note on early embryonic death. J Am Vet Med Assoc 1978;172:241e62.
[47] Nambo Y, Oikawa M, Yoshihara T, Kuwano A, Katayama Y. Age-related morphometrical changes of arteries of uterine wall in mares. Zentralblatt für Veterinarmed A 1995;42:383e7.
[48] Oikawa MKY, Yoshihara T, Kaneko M, Yoshikawa T. Microscopical characteristics of uterine wall arteries in barren aged mares. J Comp Pathol 1993;108: 411e5.
[49] R TRT. Influence of endometrial cysts on conception rate of mares. J Vet Med A 1995;A 42:275e83.
[50] Stanton MB SJPD. Endometrial cysts in the mare. J Equine Vet Sci 2004;24: 14e6.
[51] Ferreira JC, Gastal EL, Ginther OJ. Uterine blood flow and perfusion in mares with uterine cysts: effect of the size of the cystic area and age. Reproduction 2008;135:541e50.
[52] Carnevale EM, Coutinho da Silva MA, Panzani D, Stokes JE, Squires EL. Factors affecting the success of oocyte transfer in a clinical program for subfertile mares. Theriogenology 2005;64:519e27.
[53] Hoffman CEC, Costa Mattos R, Aupperle H, Dhein S, Stief B, Schoon HA. The equine endometriosis new insights into pathogenesis. Anim Reprod Sci 2009;11:261e78.
[54] Garrido-Gomez T, Ruiz-Alonso M, Blesa D, Diaz-Gimeno P, Vilella F, Simon C. Profiling the gene signature of endometrial receptivity: clinical results. Fertil Steril 2013;99:1078e85.
[55] Wu HM, Chiang CH, Huang HY, Chao AS, Wang HS, Soong YK. Detection of the subendometrial vascularization flow index by three-dimensional ultrasound may be useful for predicting the pregnancy rate for patients undergoing in vitro fertilization-embryo transfer. Fertil Steril 2003;79:507e11.
[56] Merce LT, Barco MJ, Bau S, Troyano J. Are endometrial parameters by threedimensional ultrasound and power Doppler angiography related to in vitro fertilization/embryo transfer outcome? Fertil Steril 2008;89:111e7.

Tables

Table 1
The table shows in the first column the number of embryo (0, one or two). In the second column, the number of embryos obtained per flushing performed: no embryo in 11 flushings (0 embryo/11 flushings), 26 embryos in 26 flushings (one embryo per flushing) and 14 embryos in 7 flushings (two embryos per flushings). In the third column, the ratio between the number of pregnant mares and number of embryos transferred to recipient mares are showed. And finally, the percentage of pregnancy diagnosis of recipient mares at 45 days is presented.

N° embryos	N° recovered embryos/flushing	N° pregnant mares/n° transferred embryos	Pregnancy diagnosis rate (%)
0 embryo	0/11	0	0
1 embryo	26/26	17/26	65.4
2 embryos	14/7	6/14	42.8
Total	40/44	23/40	57.5

A total of 21 flushing procedures were performed on day 7 and 23 on day 8. (Table 2).

Table 2
This table shows the day that flushing was performed (day 7 and day 8 after ovulation), the n° of positive flushings, the percentage of positive flushings, the n° of recovered embryos and the n° of flushings with twins.

Day of flushing	N° of positive flushing	Percentage of positive flushing (%)	N° recovered embryos	N° flushing with twins
D7	15/21	71.5	19	3/21
D8	18/23	78.26	21	4/23
Total	33/44	75	40	7/44

Table 3
Table showing values of area of uterine blood flow (mm^2) of pregnant and non-pregnant mares as a mean ± SEM. Mares were classified based on age range: young mares (2–5 years old), middle aged mares (6–10 years old) and old mares (>11 years old). Data were expressed as a mean ± SEM. A, b; $p \leq 0.05$.

Age	Non-pregnant mares	Pregnant mares
2–5 years old	17.21 ± 1.26^a	46.99 ± 3.41^a
6–10 years old	23.84 ± 1.50^a	60.62 ± 3.28^b
>11 years old	35.06 ± 2.56^b	67.83 ± 5.22^b

Table 4
The table show the cut-off values of endometrial blood flow area of young and adult mares on day 7 and 8 to discriminate between pregnant and non-pregnant mares. Blood flow area was expressed as a mean ± SEM.

Area of uterine blood flow(mm^2)		Day 7			Day 8		
		Mean		SEM	Mean		SEM
Young mares (2–5 years)	Non-pregnant	19.70		1.78	13.91		1.30
	Pregnant	36.29		3.28	55.03		5.04
	Cut-off values		**25.4**			**21.02**	
Adult mares (>6 years)	Non-pregnant	31.80		1.87	26.33		3.10
	Pregnant	54.06		2.98	70.31		4.78
	Cut-off values		**35.55**			**41.36**	

Figures

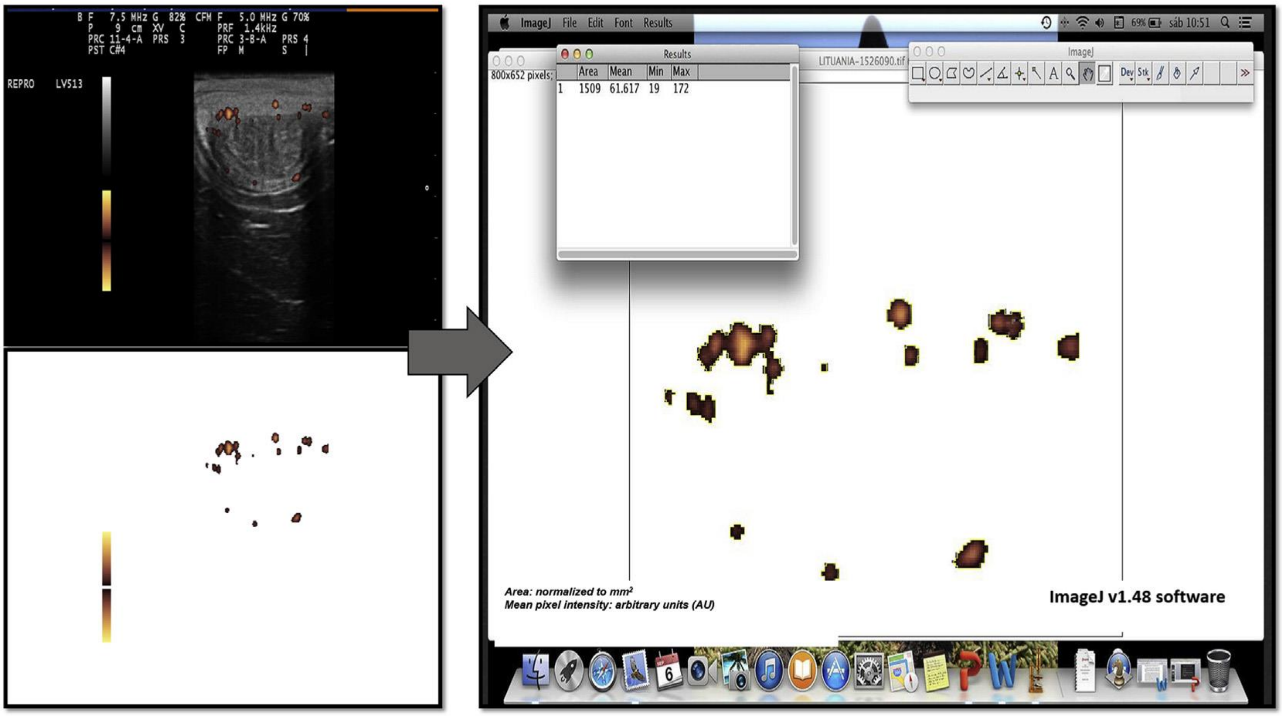


Fig. 1. Computer analysis of Doppler images using ImageJ v1.48 software (National Institutes of Health, USA). Vascular perfusion of the endometrium was assessed objectively by off-line measurement of the number of colored pixels as an indicator of blood-flow area. The blood vessels were detected in the images by applying a color threshold restricted to the uterine horn. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

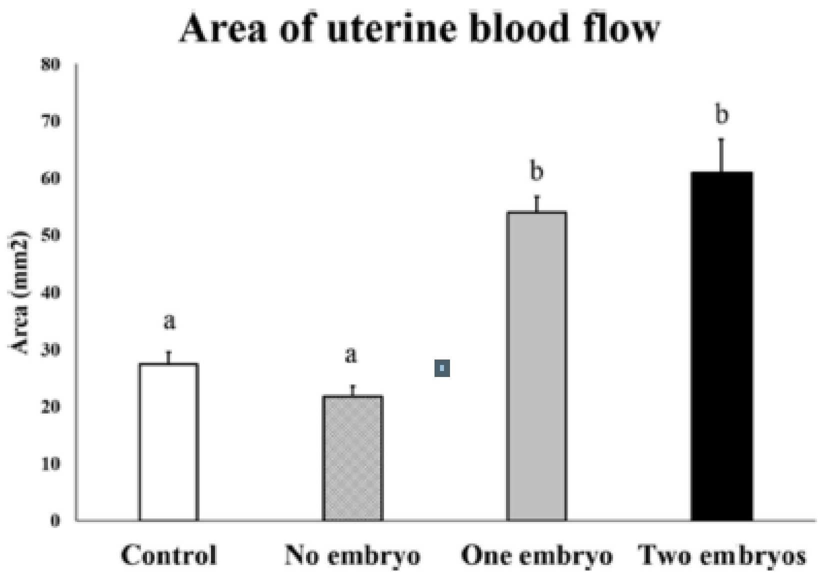
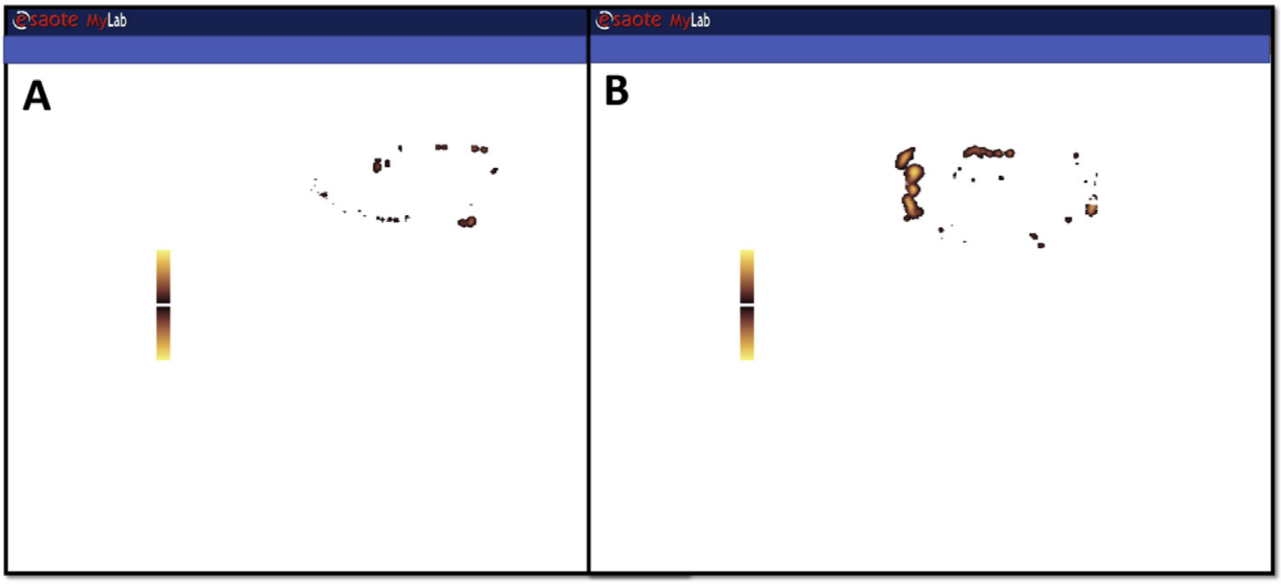


Fig. 2. This figure presents the blood flow area of the uterus of the four study's groups: Control mares (non-inseminated mare), non-pregnant mares, mares with one recovered embryo and mares with two recovered embryos. The area was expressed in mm2 as a mean ± SEM. a, b; Significance was established for $p \leq 0.05$.



Negative flushing **Positive flushing**

Fig. 3. Computer analysis of Doppler images using ImageJ v1.48 software (National Institutes of Health, USA). Examples of power Doppler images of uterine blood flow on day 7: A. Area of blood flow of a non-pregnant mare (negative flushing) and, B. Area of blood flow of a pregnant mare (positive flushing with one embryo).

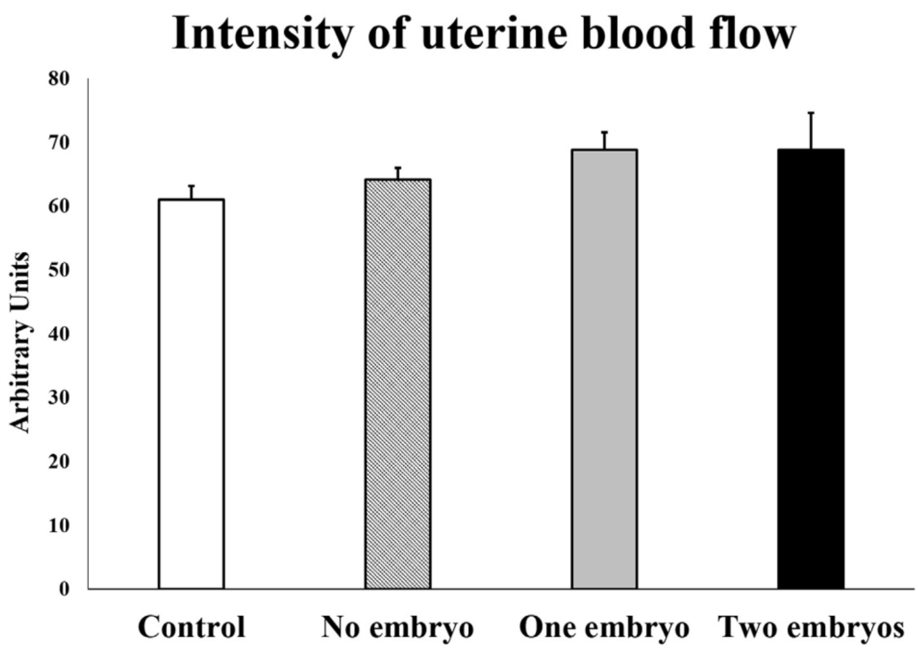


Fig. 4. This figure presents the intensity of uterine blood flow of the four study's groups: Control mares (non-inseminated mares), non-pregnant mares, mares with one recovered embryo and mares with two recovered embryos. The intensity was expressed as a mean ± SEM in arbitrary units. There were no significant differences among groups.

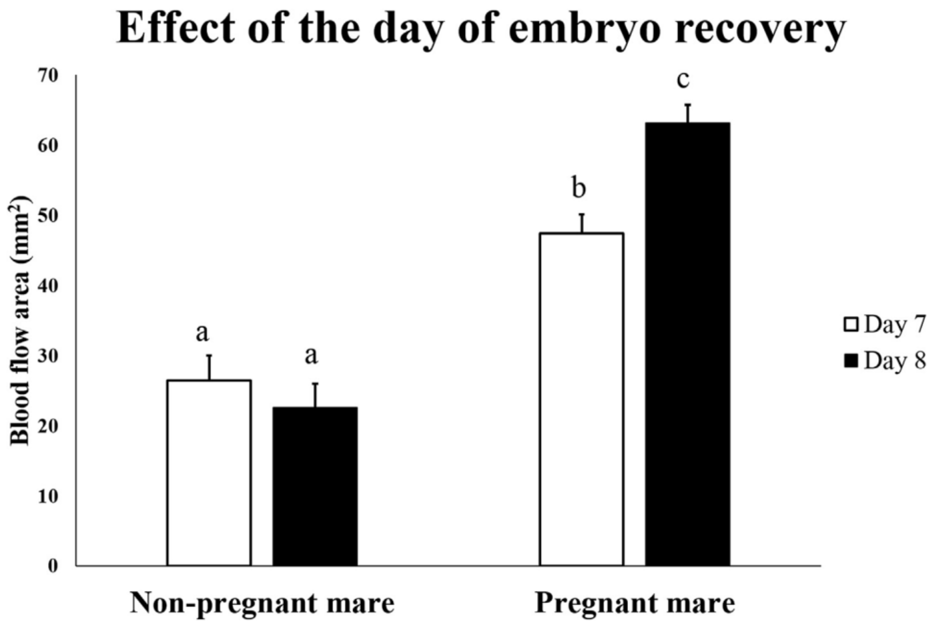


Fig. 5. This figure shows the blood flow area of uterus of pregnant and non-pregnant mares on the day 7 and day 8. The blood flow area was expressed as a mean ± SEM in mm2. a, b, c express significant differences among groups. p ≤ 0.05.

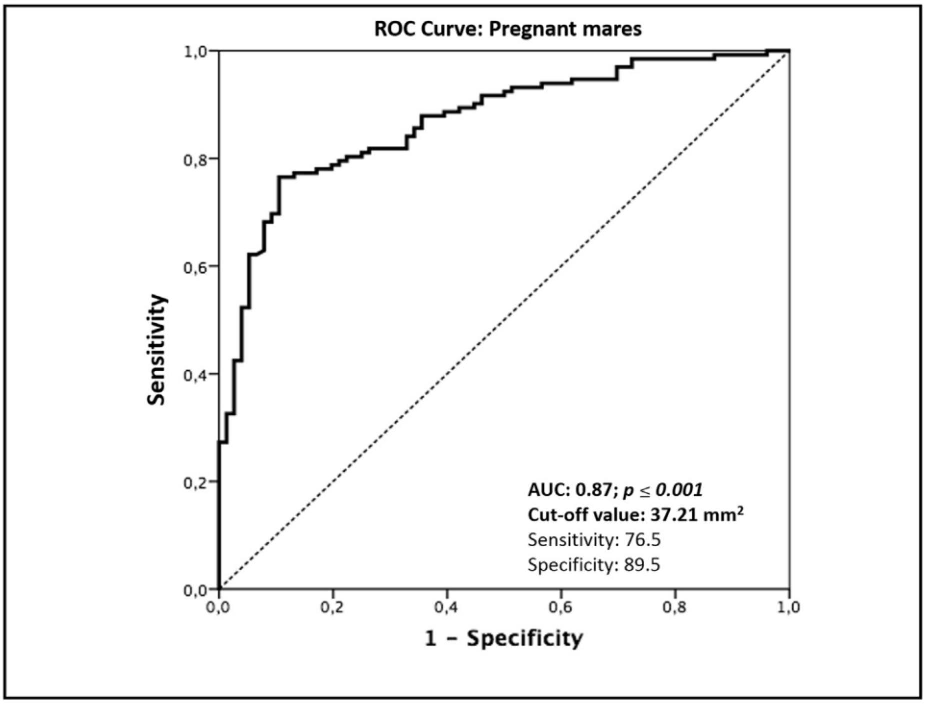


Fig. 6. Receiver operating characteristic (ROC) curves for the parameter Area of blood flow (mm2). AUC: Area under the Curve. The curve was able to detect pregnant mares, provided that the area of uterine blood flow was greater than 37.21 mm2 with a sensitivity of 76.5% and a specificity of 89.5% according to the Youden's index.

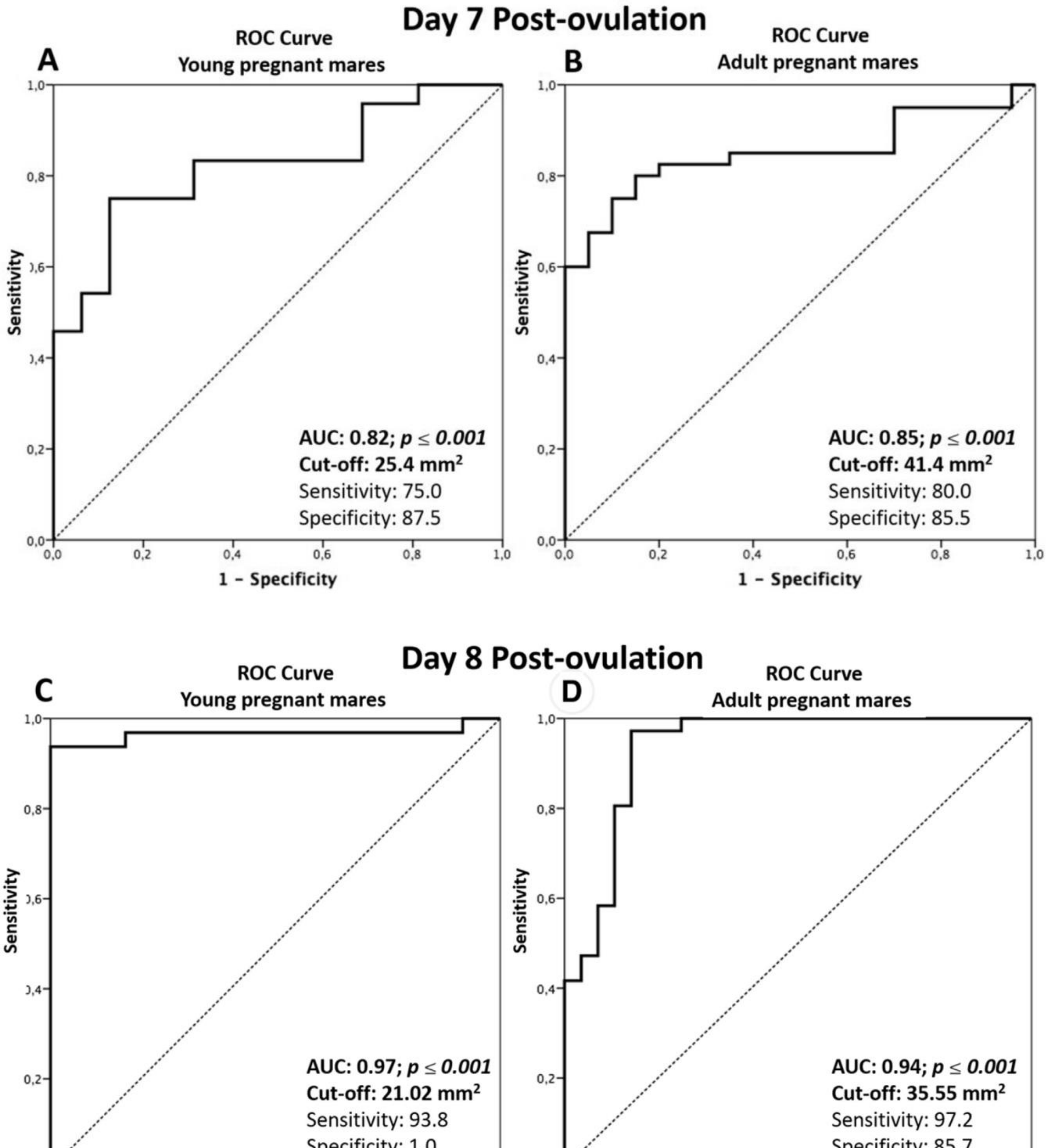


Figure 7

Fig. 7. Receiver operating characteristic (ROC) curves for the parameter of blood flow area (mm2) in young and adult mares on day 7 (A, B) and 8 (C, D) after ovulation. AUC: Area under the Curve. The curve was able to detect young pregnant mares (2e5 years old) (A, C) and adult pregnant mares (B, D) (>6 years old), provided different cut-off values of the area of uterine blood flow on day 7 and 8 after ovulation. According to Youden's test the uterine blood flow area of young pregnant mares was greater than 25.4 mm2 on day 7 (with a sensitivity of 75% and a specificity of 87.5%) and greater than 21.02 mm2 on day 8 after ovulation (with a sensitivity of 93.8% and a specificity of 100%). The uterine blood flow area of adult pregnant mares was greater than 41.4 mm2 on day 7 (with a sensitivity of 80% and a specificity of 85.5%) and greater than 35.55 mm2 on day 8 after ovulation (with a sensitivity of 97.2% and a specificity of 85.7%).