

Relationship between colour flow Doppler sonographic assessment of corpus luteum activity and progesterone concentrations in mares after embryo transfer

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ARTICLE INFO

Article history:

Received 10 September 2015

Received in revised form

18 December 2015

Accepted 22 December 2015

Available online 29 December 2015

Keywords:

Equine

Mare

Colour flow Doppler

Embryo transfer

Progesterone

ABSTRACT

Colour-flow Doppler sonography has been described as a means of assessing corpus luteum (CL) function rapidly, because area of luteal blood vessels correlates well with circulating progesterone (P_4) concentrations [P_4] in oestrous cycling mares. The aim of this study was to assess the relationships between CL size and vascularity, and circulating [P_4] during early pregnancy in mares, and to determine whether luteal blood flow was a useful aid for selecting an embryo transfer recipient. Equine embryos ($n = 48$) were recovered 8 days after ovulation and were transferred to available recipient mares as part of a commercial program with the degree of synchrony in timing of recipient ovulation ranging from 1 day before to 4 days after the donor. Immediately prior to embryo transfer (ET), maximum CL cross-section and blood vessel areas were assessed sonographically, and jugular blood was collected to measure plasma [P_4]. Sonographic measurements and jugular blood collection were repeated at day 4 after ET for all mares, and again at days 11, 18 and 25 after ET in mares that were pregnant. The number of grey-scale and colour pixels within the CL was subsequently quantified using ImageJ software. The CL blood flow correlated significantly but weakly with plasma [P_4] on the day of transfer and on day 4 after ET in all mares, and on days 11 and 25 after ET in pregnant mares ($r = 0.30$ – 0.36). The CL area and plasma [P_4] were also correlated on each day until day 11 after ET ($r = 0.49$ – 0.60). The CL colour pixel area decreased significantly after day 18, whereas CL area was already decreasing by day 4 after ET. The CL area, area of blood flow, or [P_4] was predictive of pregnancy. Findings in the present study suggest that both CL area and blood flow are correlated with circulating [P_4] at the time of transfer and in early pregnancy. Evaluation of the CL using B-mode or CF sonography, although practical, provides no improvement in the selection of recipients or prediction of pregnancy outcomes than methods employed currently.

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1. Introduction

Maintaining and managing recipient mares is the greatest recurring expense in an embryo transfer (ET)

program. However, despite attempts to select suitable recipients based on ovulation date, uterine and cervical tone (Carnevale et al., 2000) not all good quality embryos will develop into a viable pregnancy after transfer. While embryo quality is clearly an important contributor to pregnancy, and can to some extent be influenced by stallion choice and selection and management of the donor mare (Hendriks et al., 2015; Mortensen et al., 2009; Love et al.,

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2002), aspects of recipient mare management and selection could also be refined or adjusted in an effort to improve pregnancy rates. Currently, suitably oestrous synchronised mares are selected to receive an embryo based on ovulation date, uterine and cervical tone and absence of any gross abnormalities during a B-mode ultrasonic assessment of the reproductive tract.

Colour flow Doppler is an established technique for evaluating blood flow and/or patterns of vascular perfusion in human reproductive medicine (Kupesic, 1996; Fleischer, 2003) and has also been applied in studies of reproductive function in cattle (Herzog and Bollwein, 2007; Herzog et al., 2010; Lüttgenau et al., 2011; Miyamotoa et al., 2005; Utt et al., 2009), dogs (Bergeron et al., 2013; Polisca et al., 2013; Batista et al., 2013) and horses (Bollwein et al., 2001, 2002; Pozor and McDonnell, 2004; Acosta et al., 2004). Colour Doppler ultrasonography offers a reliable, non-invasive approach to evaluating vascular perfusion of the CL (Miyamotoa et al., 2005), a structure that has the greatest size-adjusted blood flow of any organ (Lüttgenau et al., 2011). Moreover, luteal blood flow is positively correlated with circulating $[P_4]$ in the non-pregnant mare, where it is a more reliable predictor of CL function than CL size, particularly during luteal regression (Bollwein et al., 2001, 2002; Ginther et al., 2007).

In practice, more than one appropriately oestrous-synchronised recipient mare maybe available for a given embryo. In general, a mare is considered suitable if she had an ovulation no more than 1 day before and no more than 3 days after the donor mare and has a tonic cervix and uterus indicative of an elevated (>1 ng/ml) circulating $[P_4]$ (Carnevale et al., 2000; McCue et al., 1999; Jacob et al., 2012). This may be supported by sonographic evidence of a CL of adequate size and homogenous echogenicity (Carnevale et al., 2000), although a mare CL with a central cavity or a less homogenous sonographic appearance can be as functional with regard to progesterone production (Townson et al., 1989). Ideally, the functionality of a CL should be evaluated by determination of an acceptable plasma $[P_4]$ (greater than 2 ng/ml; Plotka et al., 1972; Remsen et al., 1982), however, in practice plasma $[P_4]$ is rarely measured because of the added costs and the delay in ascertaining concentrations until after the time when transfers should occur. In this respect, assessment of CL blood flow would be an immediate and less expensive way of assessing CL function and thereby aid in the selecting a suitable recipient mare. The aim of the present study was, therefore, to evaluate relationships between plasma $[P_4]$ and CL size and vascularity, and to determine whether there were any cut-off values that may affect the likelihood of establishing pregnancy in mares.

2. Material and methods

2.1. Study design

The study was conducted between April and September 2013 at Utrecht University's Equine Clinic in the Netherlands. As part of the commercial clinical service, 48 embryos recovered 8 days after ovulation were transferred into reproductively sound recipient mares (age:

3–15 years). Sonographic evaluation of the recipient mare's CL using both B-mode (grey scale) and colour flow Doppler was performed on the day of embryo transfer (ET+0) and on day 4 after transfer (ET+4) when the recipient mare was also examined for pregnancy. If the mare was pregnant, the CL measurements were repeated on days 11 (ET+11), 18 (ET+18) and 25 after transfer (ET+25). Recipient mares that were not pregnant on day 4 after ET were re-examined 2 days later with no further CL measurements being performed if the mare was confirmed to be non-pregnant. At each time point the maximum cross-sectional diameter and area of colour pixels/abundance of blood vessels of the CL were recorded by one of three operators and a blood sample was collected from the jugular vein for subsequent assessment of plasma $[P_4]$.

2.2. Animals

Warmblood mares ($n=48$; 550–700 kg) with a median age of 6.5 years (range: 3–15 years) were available as recipients. Only mares receiving their first embryo of the season over which embryo transfers occurred were included in the study. All mares were free from signs of infectious disease, with grossly normal reproductive tracts and typical length oestrous cycles. The mares were maintained either on pastures with *ad libitum* access to grass and water or in stables with *ad libitum* access to hay and water. All animal procedures listed in the experimental methods were approved by Utrecht University's Animal Experimentation Committee (permission no: 2013.III.01.012).

2.3. Breeding management and oestrus synchronisation

Uterine and cervical tone were determined by rectal palpation and graded on a 1–3 point scale (1 = great tone typical of diestrus; 2 = intermediate or softening; 3 = flaccid as indicative of oestrus). Uterine oedema was graded: 1 = none, 2 = some, 3 = obvious, 4 = considerable. Follicular development and ovulation were monitored by means of trans-rectal ultrasonography. When recipient mares were in late oestrus (on the basis of a follicle ≥ 40 mm and uterine oedema >2), mares were examined daily until ovulation was detected by the disappearance of the pre-ovulatory follicle and replacement by a corpus haemorrhagicum. Mares had ovulations naturally or were induced to have an ovulation using human chorionic gonadotrophin (Chorulon®, 1500 iu per iv administration; Intervet, Boxmeer, the Netherlands). When required the luteal phase was shortened by administration of D-cloprostenol (37.5–75 μ g i.m.; Genestranvet®, Eurovet, Bladel, the Netherlands). Preferred recipient mares were those that had ovulations 0–3 days after the donor mare and if none were available, mares having ovulations 1 day before or 4 days after the donor were also considered to have sufficiently synchronised times of ovulation to receive an embryo.

2.4. Embryo recovery

Embryos were collected from donor mares by uterine lavage on day 8 after ovulation, as previously described (Stout, 2006). Embryo collection was performed using

a 37 French gauge catheter (Equine Lavage Catheter; Bioniche Animal Health, Pullman, USA) connected to silicon tubing with a Y-connector, as part of a closed system including a 75 μ m in-line embryo filter (EM CON™; Immuno Systems, Spring Valley, USA). The uterus was lavaged three times with 1 l of pre-warmed (37 °C) lactated Ringer's solution (Baxter Healthcare, Zurich, Switzerland) supplemented with 0.5% v:v foetal bovine serum (Greiner Bio One, The Netherlands). Prior to the third flushing for embryo collection, mares received 75 μ g cloprostenol intra-muscularly (Genestranvet® 75 μ g/ml, Eurovet, Bladel, the Netherlands) to assist uterine evacuation and induce luteolysis. Fluid retrieval was achieved by a combination of gravity flow and uterine massage per rectum. Upon exiting the uterus, the lavage fluid transited through the tubing to pass through the embryo filter and the contents were subsequently transferred to a petri dish.

2.5. Embryo washing, evaluation and transfer

The presence, developmental stage, size and quality of recovered embryos were determined using an Olympus SZ60 stereo dissecting microscope at 10–60 \times magnification. Once located, the embryo was washed through a five step washing procedure of increasing ratios of Syngro® Embryo Holding medium (Bioniche Animal Health, Pullman, USA) to fresh flush medium (Lactated Ringers with 0.5% v:v foetal bovine serum). The embryo was then transferred into 100% holding medium in a petri dish at ambient temperature for immediate transfer. Embryos were graded as described by McCue (2014), and then loaded individually into sterilised 0.5 ml straws and thereafter into a rigid metal ET transfer pipette with an individually packaged sterile transfer sheath and chemise (Gaine TE-J8 equine sterile; IMV Technologies, L'Aigle, France). Trans-cervical transfer into the selected recipient mare was performed using a double glove technique. Recipient mares were sedated prior to transfer with 0.3–0.4 ml detomidine hydrochloride (Domosedan® 10 mg/ml; Orion Pharma, Espoo, Finland) administered intravenously. Correct positioning of the ET pipette was determined by trans-rectal palpation of the tip of the pipette in the lumen of the uterine body or a horn, to avert accidental deposition in a cervical fold. A total of 33 grade 1 embryos, 14 grade 2 embryos, and 1 grade 3 embryos were transferred.

2.6. Data collection

2.6.1. Ultrasonography/image analysis

Ultrasonographic measurements were made using a MyLab™Five ultrasonographics machine (Esaote, Maas-tricht, The Netherlands) equipped with a 5–7.5 MHz linear array transducer. For every CL, three B-mode cross-sectional images at the site of maximal diameter were captured and stored, followed by three Colour Mode Doppler images at the maximum diameter of each CL at the time of maximum colour pixel abundance (systole). Bit map images (bmp) were saved in grey scale and colour mode at a standard depth of 10 cm, frequency of 7.5 MHz (grey scale) or 5.0 MHz (colour mode), 70% gain, and with a pulse repetition frequency of 1.0 kHz. Images were

analysed using the computer-assisted image analysis software, ImageJ (National Health Institutes, Bethesda, USA).

Using the software programme, each CL was cropped from the captured grey scale and colour Doppler images for analysis. The area and vascularisation of the CL were evaluated in a semi-quantitative manner by counting the number of grey and colour pixels, respectively. Fluid filled lacunae, if present, in a CL were outlined and excluded from analysis of the grey-scale CL pixel number. The number of grey-scale pixels was then recorded for each image of a CL and the mean of the three values used for subsequent analysis. The number of colour pixels within the CL outline in the colour flow Doppler images was assessed for each CL image and then managed as described for the grey-scale images. If two or three of the CL were present as a result of multiple ovulations in a single mare ($n = 19$ mares), the sum of the variables for all of the CL were used for analysis. All measurements were performed prior to sedation of the mares for embryo transfer.

2.6.2. Blood samples and progesterone assay

After the ultrasonic examination, jugular vein blood samples were collected into heparinised vacutainer tubes and centrifuged within 5 min at 1000 \times g for 7 min in a refrigerated centrifuge. All blood samples were collected between 10 am and 1 pm. The plasma was removed and stored at –20 °C until assay of [P₄] the [P₄] was measured using a solid-phase ¹²⁵I RIA method (Coat-A-Count TKPG; Diagnostic Products Corporation, Los Angeles, CA, USA) as described previously for the horse (Pycocock et al., 1995) as validated by Dieleman and Bevers (1987). The interassay coefficient of variation was 7.4% ($n = 5$), with sensitivity of the assay being 0.05 ng/ml.

2.7. Statistical analysis

Data analyses were conducted using the Statistical Analysis System software (SAS®, version 9.4, SAS Inst., Cary, NC, USA). Variables were tested for normal distribution using the Shapiro Wilk test. Because the data were not normally distributed, correlations between [P₄] and CL area or colour pixel area were examined using Spearman's Rank correlation. Pair-wise comparisons to assess changes over time in the measured variables were performed using the Wilcoxon signed-rank test. Data from pregnant and non-pregnant mares were compared using the Wilcoxon rank sum test for independent samples. The CL area, area of colour pixels, and [P₄] were tested using Binary logistic regression to address the possibility of them being predictors for pregnancy. All data are presented as means \pm standard deviation. Differences were considered to be significant if $P < 0.05$.

3. Results

A total of 32 of the 48 transfers (67%) resulted in pregnancy with no pregnancy losses between day 4 and 25 after transfer (i.e. the recording period). There were no differences in indices of CL size, vascularity or in plasma [P₄] between pregnant and non-pregnant recipients at days 0 and 4 after ET (Fig. 1, graphs A and B). The CL

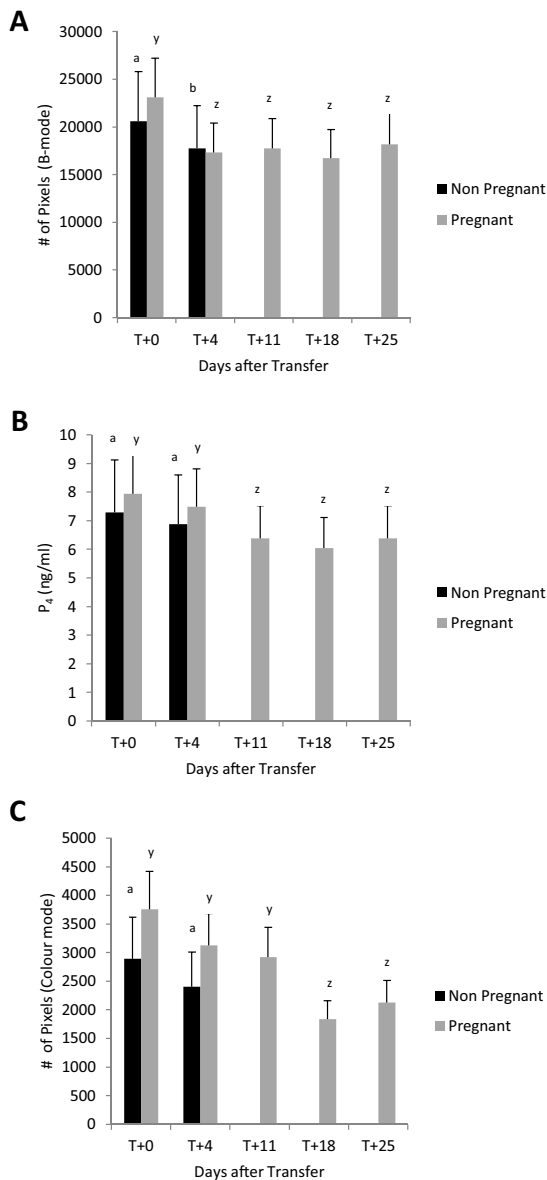


Fig. 1. Corpus luteum cross-sectional area (A, number of grey scale pixels), progesterone concentration (B, ng/ml) and vascularity (C, number of colour pixels) in recipient mares at various stages after embryo transfer. There were no significant differences ($P > 0.05$) between mares that became pregnant and those that did not. Data presented as mean values \pm standard error of the mean.

area decreased between days 0 and 4 after transfer, presumably reflecting an incompletely organised, immature early developing CL on the day of transfer. By contrast, in pregnant mares, a decrease in circulating [P₄] was noted between days 4 and 11 after transfer (Fig. 1, graph B), after which [P₄] stabilised, and the area of colour pixels/blood vessels decreased ($P < 0.05$) between days 11 and 18 after transfer (Fig. 1, graph C).

The circulating [P₄] correlated ($P > 0.001$) with CL area ($r = 0.46$) and vascularity ($r = 0.39$), as did vascularity of a CL ($P > 0.001$) with CL area ($r = 0.33$) for all data measurements.

None of CL area, area of colour pixels, or circulating [P₄] measured on the day of embryo transfer (day 0) were predictors of pregnancy outcome in recipient mares. This is not surprising given that none of the recipients involved had low [P₄] indicative of luteal failure. Variables affecting the likelihood of pregnancy in previous retrospective surveys, such as age of donor and recipient mares, degree of recipient oestrous synchrony, uterine and cervical tone, embryo grade and the use of hCG, did not influence pregnancy outcome in this prospective study, presumably because of the small number of mares/embryos included.

4. Discussion

The complete absence of blood vessels in a CL, as detected by colour flow Doppler sonography, is an indication that the CL is no longer functional, in terms of progesterone secretion. However, it appears circulating [P₄] in mares is maintained (Bollwein et al., 2002) or at least decreases more slowly during the pre-luteolytic phase (days 10–14 post ovulation; Ginther et al., 2007) than luteal blood flow. These findings suggest use of colour flow Doppler techniques could be effective in detecting a failing or regressing CL before any decrease in circulating [P₄], or changes of B-mode image or uterine/cervical tone become apparent. In the present study, the lack of a difference in circulating [P₄], CL size or blood flow between pregnant and non-pregnant mares was not surprising due to the small number of mares used, pre-selection of recipient mares for apparent reproductive normality including a normal ovulation in the oestrous cycle studied, and the fact that there is still little evidence that a greater [P₄] is more favourable for the establishment of pregnancy in mares (Willmann et al., 2011b,a). The general preference is to use recipient mares that have ovulations after the donor, meaning that first pregnancy assessments invariably occurred before the onset of cyclical luteolysis which typically begins between days 13 and 15 after ovulation with a reported range of 10–17 days (Perkins et al., 1993; Ginther et al., 2007; Bollwein et al., 2002). Possibly and more importantly, the absence of any difference in CL function between pregnant and non-pregnant mares following ET indicates that the failure to establish pregnancy was not a factor of the induction of luteolysis as a result of cervical dilation during transfer or consequent contamination as has been proposed previously (Handler et al., 2003) which is consistent with findings in a previous survey of a commercial ET programme (DeLuca et al., 2011; Handler et al., 2006).

Mares that received embryos had ovulations 4–9 days before transfer, so a decrease ($P < 0.05$) in CL area 4 days after transfer (8–13 days post recipient ovulation) is consistent with findings in a previous study (Ginther et al., 2007) where it was reported maximum cross-sectional area of the CL in mares that were not inseminated was achieved 4 days after ovulation followed by a progressive decrease to day 19 whereas plasma [P₄] peaked at day 8 before decreasing gradually to the time of onset of luteolysis. In the present study in pregnant mares, CL size remained relatively constant in size beyond day 4 after ET whereas plasma [P₄] decreased between days 4 and 11 before stabilising and CL

blood flow area did not decrease until slightly later on days 11–18. The reduction in plasma [P₄] during early pregnancy in the present study is consistent with results of previous reports that progesterone production peaks in pregnant mares (12–20 ng/ml) between days 5 and 10 after ovulation and then decreases steadily to values as low as 3–5 ng/ml by day 35 (Allen, 2001). The rate of decrease in CL vascularity was greater during the pre-luteolytic period (days 10–14) than the decrease in progesterone in non-pregnant mares in previous research (Ginther et al., 2007). In the current study, this pattern was reversed (*i.e.* plasma [P₄] began to decrease before CL vascularity) perhaps in part reflecting the influence of pregnancy.

Reports on the reliability of luteal blood flow as an indicator of CL function in terms of circulating [P₄] are inconsistent with some studies involving dairy cattle where it was reported that luteal blood flow was a superior predictor of [P₄] than luteal size (Herzog et al., 2010) whilst other reports indicate there is no association between these two variables (Lüttgenau et al., 2011). With regard to mares, colour Doppler measurements parallel circulating [P₄] in non-pregnant mares at the time of luteolysis, as CL size decreases earlier, but there is no definitive evidence that it is a more reliable predictor of [P₄] than luteal size (Bollwein et al., 2002; Ginther et al., 2007). In the present study, both CL area ($r=0.45$) and vascularity ($r=0.39$) were correlated ($P>0.0001$) with plasma [P₄]. It is, therefore, concluded that both the B-mode ultrasonographic evaluation of luteal size and colour flow Doppler assessment of blood area are equally useful indicators of CL function in pregnant mares.

In conclusion, transrectal grey-scale and colour Doppler sonography can be easily assessed and are non-invasive methods for examining luteal function in pregnant mares. Colour Doppler ultrasonography might be particularly useful for identifying individual mares where luteolysis has been initiated, given that CL blood flow decreases more rapidly after the initiation of luteolysis than CL area (17.3% per day compared with 7.7% per day) (Ginther et al., 2007). Nevertheless, the use sonographic assessment of luteal function in the selection and monitoring of recipient mares is likely to do no more than identify the occasional mare with a clearly inadequate CL at the time of transfer, or the small number of mares that are pregnant but return to oestrus as a result of failed maternal pregnancy recognition or luteolysis that is initiated by other factors. There is no reason to suggest that selecting potential recipient mares on the basis of a larger or more vascular CL is likely to improve results from embryo transfer in mares.

Conflict of interest

None.

Acknowledgement

All funding and support for this project was provided by the University of Utrecht Residency Training Program.

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