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Early pregnancy diagnosis in ewes by subjective assessment of luteal vascularisation using colour Doppler ultrasonography

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ABSTRACT

This study was designed to evaluate early pregnancy diagnosis using colour Doppler ultrasonography (US) for luteal vascularisation assessment. In Study 1, 28 ewes were artificially inseminated (Day 0), and luteal vascularisation was assessed from Day 12 to Day 19 by two evaluators using colour Doppler US, categorising the corpus luteum (CL) on a subjective scale ranging from 1 to 4. Females bearing a CL with score 2 or greater were presumably considered pregnant. Pregnancy was confirmed on Day 30 by B-Mode US. In Study 2, a predictive pregnancy diagnosis was performed on Day 17 in 197 ewes based on the criteria described in Study 1. Pregnancy was confirmed by B-mode US on Day 45. Agreement between evaluators was verified by an intraclass correlation coefficient (ICC) and Kappa index (κ). Performance of colour Doppler US for early pregnancy diagnosis was evaluated calculating sensitivity (Sens), specificity (Spec), negative predictive values (NPV), positive predictive values (PPV) and accuracy (Ac). In Study 1, luteal vascularisation assessment was unable to predict non-pregnant animals between 12 and 14 days after insemination, as all animals still had vascularised CL, and thus were considered pregnant. The colour Doppler US performance improved progressively until Day 17, when it reached maximum values (Sens = 100%, Spec = 76%, PPV = 73%, NPV = 100% and Ac = 86%). The subjective scale for luteal irrigation assessment showed medium to good agreement among evaluators on Day 12 and Day 13 (ICC = 0.66 and 0.68, respectively), and excellent agreement from Day 14 to Day 19 (ICC = 0.90, 0.80, 0.80, 0.84, 0.95 and 0.93, respectively). Agreement was almost perfect for score 1 CLs (κ = 0.87), and moderate for scores 2, 3 and 4 CLs (κ = 0.54, 0.48 and 0.41, respectively). In Study 2, performance of colour Doppler US as a tool to predict pregnancy status in ewes on Day 17 post-insemination was as follows: Sens = 93.5%, Spec = 80.8%, PPV = 85.6%, NPV = 91.1% and Ac = 87.8%. Subjective luteal vascularisation assessment using colour Doppler US to distinguish between pregnant and non-pregnant animals was considered a reliable tool which was highly efficient beginning 17 days after breeding.

1. Introduction

Pregnancy diagnosis is a critical step of the reproductive management of any animal production system. Identification of non-pregnant females is important for decision-making regarding rebreeding or culling of those animals. The earlier that pregnancy can be diagnosed, the earlier decisions can be made; thus, an early, accurate and feasible pregnancy diagnosis method is essential for optimising reproductive performance. Observation of oestrus behaviour after breeding is the simplest and possibly the most widely used method to detect non-pregnant animals, but in ewes, the oestrous cycle length has a wide range of 14–19 days [1,2]. Moreover, pregnancy diagnosis based on detection of oestrus signs needs the use of teaser animals, and usually is laborious. Moreover, this method is not useful for out-of–season breeding, as many ewes

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come into oestrus only once even if they are not pregnant [3]. Measurement of blood progesterone concentration is 100% accurate for detecting non-pregnant animals from days 16–18 post-breeding [4,5], but is highly expensive, requires laboratory support and does not provide real-time results. Hence, it is not a practical tool for reproductive management under field conditions [6].

Ultrasonography (US) is a safe non-invasive technique that provides real-time results based on direct interpretation of images. Currently, this imaging technique is the most widely used method for pregnancy diagnosis under field conditions. In ovine, although pregnancy might be recognized as early as 17–19 days post-breeding using B-Mode US, it can take at least 25 days after oestrus for accurate and reliable results [7]. The potential of colour Doppler US for reproductive management in farm animals was initially demonstrated in the early 2000s [8,9]. Since then, several studies have been conducted demonstrating that colour Doppler US can be successfully used to evaluate vascularisation of several reproductive organs and structures [10–12], including the corpus luteum [13]. Luteal vascularisation acute decreases simultaneously with a decrease in the concentration of blood progesterone, indicating that the luteolysis process has begun [14], and consequently that fertilisation or the pregnancy establishment process failed to occur. In bovine and ovine, luteal vascularisation significantly decreases 24 h after the onset of the luteolytic process [15,16], and it has been demonstrated that subjective assessment of luteal vascularisation using colour Doppler US is a potential tool for early pregnancy diagnosis. In bovine, non-pregnant cows were effectively identified as early as 20 days after breeding [17]. In ovine, however, the effectiveness of a subjective assessment of luteal vascularisation as a tool for early pregnancy diagnosis, determining the earliest post-breeding time to detect non-pregnant animals, has not been determined.

The objective of the present study was: (1) evaluate the efficiency of a subjective luteal vascularisation assessment using colour Doppler US for early pregnancy diagnosis, (2) determine the earliest post-breeding time at which this evaluation is effective, and (3) validate its use for practical pregnancy diagnosis in sheep flocks.

2. Materials and methods

In order to achieve the proposed objectives two independents but complementary studies were conducted. All procedures performed were approved by the Ethical Committee for Animal Use of the Universidade Federal Fluminense (protocol #923/2017) and were carried out under the ethical principles of the Conselho Nacional de Controle de Experimentação Animal (CONCEA).

2.1. Study 1

Study 1 was conducted at Unidade de Pesquisa Experimental em Caprinos e Ovinos of the Universidade Federal Fluminense, located in Cachoeiras de Macacu (22°S, 42°W), Rio de Janeiro, Brazil, with 28 adult Santa Ines ewes [age: 3.0 ± 1.2 years-old, body weight: 44.6 ± 5.4 kg, body condition score: 3.0 ± 0.2 (scale 1–5; [18]) (mean ± SD)]. All animals were submitted to a gynaecological exam before the study, and only animals without reproductive abnormalities detected by ultrasonography or clinical exam were used. Throughout the study, ewes were kept in a grazing system (*Brachiaria decumbens*), and supplemented with chopped grass (*Pennisetum purpureum*) and concentrate according to maintenance requirements [19]. Water and mineral salt were provided *ad libitum*. After an oestrous synchronisation protocol, all animals were artificially inseminated (Day 0), and subjective luteal vascularisation assessment for pregnancy diagnosis using colour Doppler US was performed daily from Day 12 to Day 19 and confirmed by B-Mode US on Day 30.

2.1.1. Oestrus synchronisation and fixed-time artificial insemination (FTAI)

A sponge impregnated with 60 mg of medroxyprogesterone acetate (Progespon, Schering Plough, São Paulo, Brazil) was inserted in each animal, and remained in situ for 6 days. Twenty-four hours before sponge withdrawal, all animals received 0.24 mg of cloprostenol sodium (Estron, Agner União, São Paulo, Brazil) and 300 IU of equine chorionic gonadotropin (Novormon, Schering Plough, São Paulo, Brazil). Thirty-six hours after sponge withdrawal, 0.025 mg of gonadorelin acetate was administrated to each ewe (Gestran Plus, Tecnopec, São Paulo, Brazil).

Intra-uterine insemination was performed 56 h after sponge withdrawal by laparoscopy, as previously described [20]. Ewes were subjected to fasting from food and water for 24 h and 12 h before laparoscopic artificial insemination (AI), respectively. Pre-anesthetic treatment was administered 15 min before the AI procedure using 0.1 mg/kg of acepromazine iv (Acepran, Vetnil, São Paulo, Brazil), 0.5 mg/kg of diazepam iv (Diazepan, Teuto, Goiás, Brazil) and 0.4 mg/kg of morphine im (Dolo Moff, União Química, São Paulo, Brazil). Then, animals were positioned in the Trendelenburg position, and lidocaine 2% sc (Lidovet, Bravet, Rio de Janeiro, Brazil) was administered in the region of trocars insertion. Insemination was performed using commercial frozen-thawed semen straw containing 40×10^6 spermatozoa (0.25 mL straw thawed at 35 °C for 30 s) from three Santa Inês rams of proven fertility.

2.1.2. Colour Doppler ultrasound and early pregnancy prediction

After FTAI, ultrasonographic observations of the ovaries were performed every 24 h from Day 12 to Day 19 using portable equipment (Sonoscape S6, Shenzhen, China) with a 7.5 MHz linear rectal transducer. First, the ovary bearing the CL was located by B-Mode US. Thereafter, colour flow mode (CFM) was activated, and a complete scan of the CL was performed. Luteal vascularisation was evaluated using a subjective scale with scores ranging from 1 to 4 (Fig. 1). During a complete scan of the CL, the proportion of the luteal area with coloured pixels was subjectively estimated and then scored as 1 (0–25%), 2 (26–50%), 3 (51–75%) or 4 (76–100%). After both ovaries were scanned, it was assumed that animals bearing at least one CL with a vascularisation score ≥ 2 were pregnant. Each exam using CFM was video-recorded and stored within the equipment's internal hard drive. All examinations were performed by an experienced technician. Doppler settings were standardised and maintained constant in all recordings (CFM frequency: 6.0 MHz, Pulse Repetition Frequency [PRF]: 1.0 KHz, and Wall Filter [WF]: 75 KHz). The videos previously recorded were evaluated by a second experienced technician. Identification of the animals was blinded for this second evaluator, who performed the luteal vascularisation assessment and predictive pregnancy diagnosis as previously described. Pregnancy was diagnosed on Day 30 based on the detection of a viable embryonic vesicle within the uterine horn using B-Mode US. Pregnancy viability was confirmed by the presence of an embryo heartbeat.

Pregnancy predictions based on the evaluators' luteal vascularisation assessments from Day 12 to Day 19 were related to results obtained on Day 30, which were considered the gold standard, and classified as follows: true positive (TP, animals diagnosed as pregnant by both methods), true negative (TN, animals diagnosed as non-pregnant by both methods), false positive (FP, animals diagnosed as pregnant by luteal vascularisation assessment, but as non-pregnant by B-Mode US on Day 30) or false negative (FN, animals diagnosed as non-pregnant by luteal vascularisation assessment,

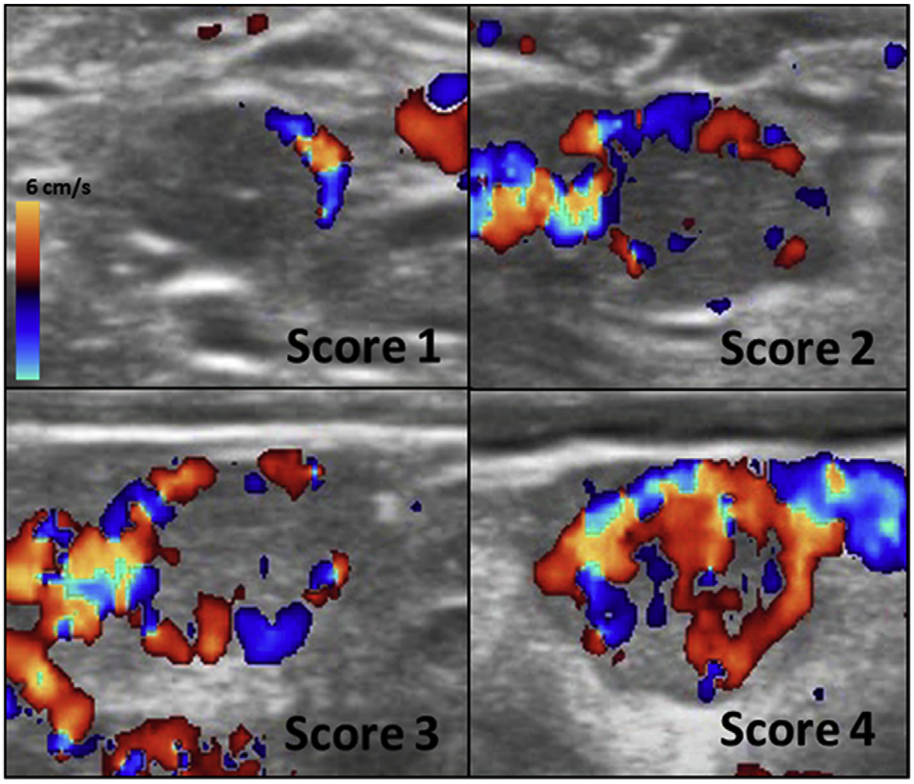

Fig. 1. Ultrasonographic images demonstrating the score scale used for subjective luteal vascularisation assessment.

but as pregnant by B-Mode US on Day 30).

2.2. Study 2

Study 2 was conducted at Estación Experimental Bernardo Rosengurtt of Facultad de Agronomía, Universidad de la República, located in Cerro Largo (32°S, 54°W), Uruguay with 197 adult Corriedale ewes [age: 4.0 ± 1.5 years-old, body weight: 45.6 ± 6.7 kg, body condition score: 3.5 ± 0.8 (scale 1–5, [18])]. Animals grazed under extensive conditions on natural pastures at approximately 3.0 kg DM/kg of their live weight, and they had free access to water. Oestrus were synchronised as part of another study, and all animals were artificially inseminated, and subjective luteal vascularisation assessment for pregnancy diagnosis using colour Doppler US was performed on Day 17. Pregnancy was later confirmed by B-Mode US on Day 45.

2.2.1. Artificial insemination

Oestrus was recorded twice daily with vasectomised rams, and ewes were inseminated 12 h after oestrus onset with fresh semen collected from two fertile Corriedale rams. Semen doses of approximately 2×10^8 spermatozoa in 0.2 mL of volume were inserted using a speculum equipped with a light source and a multidose insemination gun (Walmur Veterinary Instrument, Montevideo, Uruguay).

2.2.2. Colour Doppler ultrasonography and early pregnancy prediction

Ovaries were scanned 17 days after insemination using a portable device (MyLabOne, Genoa, Italy) equipped with a 7.5 MHz linear rectal transducer. Similar to what was described in Study 1, the ovary bearing the CL was first located using B-Mode US; then CFM was activated and a complete scan of the CL was performed with the same CFM frequency, PRF and WF used in Study 1. Luteal vascularisation was assessed and predictive pregnancy diagnosis was performed, as in Study 1. All sonograms and pregnancy diagnoses were performed by the same experienced technician of Study 1. Pregnancy was confirmed by B-mode US on Day 45 after AI. Pregnancy predictions by luteal vascularisation assessment that were performed on Day 17 were related to results that were obtained on Day 45 and, as previously described, were classified as true positive (TP), true negative (TN), false positive (FP) or false negative (FN).

2.3. Data analysis

In Study 1, the agreement between both evaluators using the subjective luteal vascularisation score system was analysed by the Intraclass Correlation Coefficient (ICC) from Day 12 to Day 19, and by the Kappa index (κ) for each vascularisation score. By using ICC analysis, reproducibility of the score system was classified as low ($ICC < 0.40$), medium to good ($0.41 \leq ICC \leq 0.75$) or excellent ($ICC > 0.75$), according to [21]. By κ analysis, agreement between the evaluator and between colour Doppler and B-Mode US was classified as poor ($\kappa < 0.00$), slight ($0.00 < \kappa < 0.20$), fair ($0.21 < \kappa < 0.40$), moderate ($0.41 < \kappa < 0.60$), substantial ($0.61 < \kappa < 0.80$) or almost perfect ($0.81 < \kappa < 1.00$), according to [22]. From Day 12 to Day 19, the performance of subjective luteal vascularisation assessment using colour Doppler US as a tool for early pregnancy diagnosis was evaluated by calculating the following parameters: Sensitivity (Sens), Specificity (Spec), Positive Predictive Value (PPV), Negative Predictive Value (NPV), Accuracy (Ac), Positive Likelihood Ratio (LR+) and Negative Likelihood Ratio (LH-). The following equations were used to calculate each parameter: Sens = TP/(TP + FN), Spec = TN/(FP + TN), PPV = TP/(TP + FP), NPV = TN/(FN + TN), Ac = (TP + TN)/n, LR+ = Sens/100-

Spec and LR- = 100-Sens/Spec. ICC and κ index were calculated using the software BioEstat 5.3.

The same parameters were calculated in Study 2 to determine the efficiency of subjective luteal vascularisation assessment using colour Doppler US as a tool for early pregnancy diagnosis on Day 17. Additionally, the intensity of agreement between both techniques (colour Doppler US and B-Mode US) was evaluated by Kappa index.

3. Results

In Study 1, the agreement between evaluators on the subjective score scale to assess luteal vascularisation was medium to good at Day 12 and Day 13, and excellent from Day 14 to Day 19 (Table 1). The overall agreement between evaluators was moderate (κ = 0.59). When each score was evaluated separately, the best agreement between evaluators was observed for score 1 (κ = 0.87). This agreement progressively decreased from score 2 to score 4 (Table 2).

The overall performance of subjective luteal vascularisation assessment using colour Doppler US as a tool for early pregnancy diagnosis at different moments post-insemination is presented in Table 3. Use of colour Doppler US from Day 12 to Day 14 was infeasible (all animals still had well vascularised CL). Throughout this period, all animals diagnosed as non-pregnant on Day 30 were predictively classified as pregnant by both evaluators, except for one animal that was incorrectly classified as non-pregnant on Day 12 by the second evaluator. From Day 15 to Day 17, overall performance of colour Doppler US as a tool for early pregnancy diagnosis progressively increased, and results did not change from Day 17 to Day 19. Furthermore, four animals diagnosed as non-pregnant on Day 30 had a well vascularised CL until Day 19.

In Study 2, the number of TP, FP, TN and FN results were 101, 17, 72 and 7 ewes, respectively. Thus, the performance of colour Doppler US as a tool to predict pregnancy status in ewes on Day 17 post-insemination was 93.5% (Sens), 80.8% (Spec), 85.5% (PPV), 91.1% (NPV), 87.8% (Ac), 4.9 (95% CI, 3.18–7.53; LR+), and 0.08 (95% CI, 0.04–0.17; LR-). Colour Doppler US on Day 17 post insemination had a substantial agreement (κ = 0.75) using the gold standard method (B-Mode US on Day 45) for pregnancy diagnosis.

4. Discussion

Colour Doppler US resulted in an excellent tool for early pregnancy diagnosis in ewes. In both studies, pregnancy was determined with high accuracy in ewes as early as 17 days after oestrus, which may allow development of strategies for more intense reproductive management, such as early re-synchronisation of oestrus in non-pregnant ewes, saving almost 2 weeks compared with early pregnancy diagnosis done by transrectal classical ultrasound [23]. In addition, colour Doppler US may be very useful for reproductive management for out-of-season breeding, as pregnancy diagnosis based on the return of oestrus signs cannot be reliably performed [24]. Therefore, colour Doppler US in sheep is useful not only for health diagnoses [25], but also has a direct positive impact on productive and reproductive management decisions. This finding in studies with sheep is similar to previous results reported in bovine, in which non-pregnancy in animals could be successfully detected 20–22 days post-breeding [17].

The main application of early pregnancy diagnosis is the identification of non-pregnant animals [26]. Thus, determination of the earliest moment at which the use of subjective luteal vascularisation assessment is more efficient was mainly based on NPV and specificity results. In sheep, the luteolytic process starts between Days 13 and 16 of the oestrous cycle [27], and luteal vascularisation significantly decreases during the first 24 h after luteolysis begins [16]. Therefore, in Study 1, it was observed that detection of non-pregnant animals based on subjective luteal vascularisation assessment using colour Doppler US was infeasible from Day 12 to Day 14 because most animals still had vascularised CL (the pre-luteolysis period). From Day 15 onward, all animals diagnosed as non-pregnant using colour Doppler US were later confirmed as not pregnant by B-Mode US (FN = 0 and NPV = 100%). However, the specificity of the proposed methodology did not reach its maximum value until Day 17 (76.5%), when the FP results reached their minimum values. When the proposed methodology was evaluated as a practical tool for pregnancy diagnosis on Day 17 post-insemination (Study 2), most non-pregnant animals were correctly identified as in Study 1 (FN = 7, NPV = 91.1% and Spec = 80.8%), although confirmation of pregnancy in Study 2 was made on Day 45 post-insemination, and in Study 1 on Day 30. In both studies, FP was the most commonly observed error. False positive results may be explained by the variability in the moment that luteolysis begins, or by the occurrence of early embryo mortality, which can lengthen the oestrous cycle [28]. Previous studies in domestic ruminants showed that the incidence of embryo loss is between 12% and 30% [28, reviewed by 29]. In sheep, the occurrence of embryo loss between days 26 and 32 post-mating was 17.2%, but could reach up to 30% in ewes submitted to stressful management [30]. The occurrence of a short (<14 days) or long (>19 days) oestrous cycle may also lead to FP results. In a previous study with Santa Inês, Suffolk and Romney Marsh ewes, the incidence of short and long oestrous cycles was not affected by the breed and was observed in less than 15% of the evaluated oestrous

Table 2
Agreement between both evaluators for each score of luteal vascularisation.

Luteal vascularisation score	Kappa index	Interpretation
1	0.87	Almost perfect agreement
2	0.54	Moderate agreement
3	0.48	Moderate agreement
4	0.41	Moderate agreement

Table 1
Intraclass correlation coefficient (ICC) between first and second evaluators for subjective luteal vascularisation scoring at different moments after fixed-time artificial insemination.

Time after insemination (days)	ICC (95% CI[a])	Reproducibility Interpretation
12	0.66 (0.39–0.82)	Medium to Good
13	0.68 (0.42–0.82)	Medium to Good
14	0.90 (0.80–0.94)	Excellent
15	0.80 (0.63–0.89)	Excellent
16	0.80 (0.62–0.89)	Excellent
17	0.84 (0.70–0.91)	Excellent
18	0.95 (0.90–0.97)	Excellent
19	0.93 (0.86–0.96)	Excellent

[a] 95% confidence interval.

Table 3
Overall performance of subjective luteal vascularisation assessment using colour Doppler ultrasonography for early pregnancy diagnosis from Days 12–19 post-insemination.

Time after insemination (days)	Evaluator n°1							
	TP, FP, FN, TN (n)	Sens (%)	Spec (%)	PPV (%)	NPV (%)	Ac (%)	LR+	LR-
12	11, 17, 0, 0	100	0	39.3	NC	39.2	1	NC
13	11, 17, 0, 0	100	0	39.3	NC	39.2	1	NC
14	11, 17, 0, 0	100	0	39.3	NC	39.2	1	NC
15	11, 14, 0, 3	100	17.6	44.0	100	50.0	1.2	0
16	11, 9, 0, 8	100	47.1	55.0	100	67.8	1.9	0
17	11, 4, 0, 13	100	76.5	73.3	100	85.7	4.3	0
18	11, 4, 0, 13	100	76.5	73.3	100	85.7	4.3	0
19	11, 4, 0, 13	100	76.5	73.3	100	85.7	4.3	0
Time after insemination (days)	**Evaluator n°2**							
	TP, FP, FN, TN (n)	**Sens (%)**	**Spec (%)**	**PPV (%)**	**NPV (%)**	**Ac (%)**	**LR+**	**LR-**
12	10, 16, 1, 0	91.0	0	38.0	0	35.7	0.90	NC
13	11, 17, 0, 0	100	0	39.3	NC	39.2	1	NC
14	11, 17, 0, 0	100	0	39.3	NC	39.2	1	NC
15	10, 13, 0, 4	100	23.5	43.5	100	50.0	1.3	NC
16	11, 6, 0, 11	100	64.7	64.7	100	78.5	2.8	0
17	11, 4, 0, 13	100	76.5	73.3	100	85.7	4.3	0
18	11, 4, 0, 13	100	76.5	73.3	100	85.7	4.3	0
19	11, 4, 0, 13	100	76.5	73.3	100	85.7	4.3	0

TP, True positive; *FP*, False positive; *FN*, False negative; *TN*; True negative; *Sens*, Sensitivity; *Spec*, Specificity; *PPV*, Positive predictive value; *NPV*, Negative predictive value; *Ac*, Accuracy; *LR+*, Positive likelihood ratio; *LR-*, Negative likelihood ratio.
NC – not calculable.

cycles [31]. On the other hand, the length of the oestrous cycle can be affected by parity, as hoggets' oestrous cycles are shorter than those of adult ewes [32]. The number of FP results in the present study was within the expected range; however, factors that can increase the number of FP results must be practically considered in reproductive programs.

In Study 1 we demonstrated that although luteal vascularisation was assessed using a subjective score scale, there was moderate to high agreement between evaluators, and that this agreement progressively increased from Day 12 to Day 19 post-breeding. This progressive increase in ICC values can be explained by the differences observed when the agreement between evaluators was separately evaluated for each score category. As observed for subjective embryo quality assessment [33], the agreement was not similar among CLs of different scores. Distinction among CLs with vascularisation scores 2, 3 and 4 was more difficult than those with vascularisation score 1. Considering the normal activity of CL during the late luteal phase of non-pregnant ewes, no CL vascularisation was given score 1 by both evaluators until Day 15. As the proportion of CLs categorised as score 1 increased due to the luteolytic process, the agreement between evaluators also increased. Considering that the main objective of early pregnancy diagnosis is to identify non-pregnant animals, i.e. animals bearing a CL with vascularisation score 1, this difficulty in distinguishing between scores 2, 3 and 4 is not a limiting factor for applying the technique.

The pregnancy diagnosis methodology proposed in the present study achieved the practical requirements to be used under field conditions, i.e., it was efficient, quick and provided real-time results. The correct identification of non-pregnant animals as early as 17 days post-breeding opens the possibility to develop hormonal protocols to quickly re-synchronize these animals, reducing interval between inseminations. Possible increases in accuracy depend on complementary analysis of the images obtained by coloured Doppler US, but this limits the applicability of the technique, as results cannot be obtained in real time. Determination of blood flow velocity is not possible due to the small size and/or tortuosity of the blood vessels present in the female reproductive tract [34]. Three-dimensional evaluation [35], and calculation of the proportion or area of coloured pixels or the estimation of the number of coloured pixels provide interesting results [16,36–39], but requires a post-processing image procedure, and thus not allowing real-time results.

Overall, we concluded that the subjective assessment of corpus luteum irrigation by colour Doppler US can be effectively used for pregnancy diagnosis as early as 17 days after breeding.

Authors' contributions

FZB, RU and RPC conceived the project and coordinated the study. All authors contributed to the experimental activities. FZB, RU, RPC and EKNA drafted the manuscript, which was revised by all authors. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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References

[1] Baird DT, Swanston I, Scaramuzzi EJ. Pulsatile release of LH and secretion of ovarian steroidsin sheep during the luteal phase of the estrous cycle. Endocrinology 1976;98:1490–6.
[2] Senger PL. Pathways to pregnancy and parturition. 2nd revised ed. Washington: Current conceptions, Inc; 2005.
[3] Ungerfeld R, Suárez G, Carbajal B, Silva L, Laca M, Forsberg M, et al. Medroxyprogesterone primings and response to the ram effect in Corriedale ewes during the non-breeding season. Theriogenology 2003;60:35–45.
[4] McPhee IM, Tiberghien MP. Assessment of pregnancy in sheep by analysis of

plasma progesterone using an amplified enzyme immunoassay technique. Vet Rec 1987;121:63–5.
[5] Susmel P, Piasentier E. Assessment of pregnancy in Bergamasca ewes by analysis of plasma progesterone. Small Rum Res 1992;8:325–32.
[6] Barbagianni MS, Ioannidi KI, Vasileiou NGC, Mavrogianni VS, Orfanou DC, Fthenakis GC, et al. Ultrasonographic examination of pregnant ewes: from early diagnosis of pregnancy to early prediction of dystocia. Small Rumin Res 2017;152:41–55.
[7] García A, Neary MK, Kelly GR, Pierson RA. Accuracy of ultrasonography in early pregnancy diagnosis in the ewe. Theriogenology 1993;39:847–61.
[8] Bollwein H, Meyer HH, Maierl J, Weber F, Baumgartner U, Stolla R. Transrectal doppler sonography of uterine blood flow in cows during the estrous cycle. Theriogenology 2000;53:1541–52.
[9] Miyamoto A, Shirasuna K, Hayashi KG, Kamada D, Awashima C, Kaneko E, et al. A potential use of color ultrasound as a tool for reproductive management: new observations using color ultrasound scanning that were not possible with imaging only in black and white. J Reprod Dev 2006;52:153–60.
[10] Oliveira ME, Feliciano MA, D'Amato CC, Oliveira LG, Bicudo SD, Fonseca JF, et al. Correlations between ovarian follicular blood flow and superovulatory responses in ewes. Anim Reprod Sci 2014;144:30–7.
[11] Ginther OJ. How ultrasound technologies have expanded and revolutionized research in reproduction in large animals. Theriogenology 2014;81:112–25.
[12] Beltrame RT, Covre C, Littig LB, Martins AB, Quirino CR, Junior AB, et al. Transrectal Doppler sonography of uterine blood flow in ewes during pregnancy. Theriogenology 2017;91:55–61.
[13] Acosta TJ, Miyamoto A. Vascular control of ovarian function: ovulation, corpus luteum formation and regression. Anim Reprod Sci 2004;82–83:127–40.
[14] McCracken JA, Custer E, Lamsa JC. Luteolysis: a neuroendocrine-mediated event. Physiol Rev 1999;79:263–323.
[15] Acosta TJ, Yoshizawa N, Ohtani M, Miyamoto A. Local changes in blood flow within the early and midcycle corpus luteum after prostaglandin F(2alpha) injection in the cow. Biol Reprod 2002;66:651–8.
[16] Figueira LM, Fonseca JF, Arashiro EKN, Souza-Fabjan JMG, Ribeiro ACS, Oba E, et al. Colour Doppler ultrasonography as a tool to assess luteal function in Santa Inês ewes. Reprod Dom Anim 2015;50:643–50.
[17] Siqueira LGB, Areas VS, Ghetti AM, Fonseca JF, Palhao MP, Fernandes CAC, et al. Color Doppler flow imaging for the early detection of nonpregnant cattle at 20 days after timed artificial insemination. J Dairy Sci 2013;96:6461–72.
[18] Suiter J. Body condition scoring of sheep and goats. Farmnote 1994;69:1–4.
[19] NRC – NATIONAL RESEARCH COUNCIL. Nutrient requirements of small ruminants: sheep, goats, cervids and new world camelids. Washington: National Academy Press; 2007. 362pp.
[20] Teixeira TA, Fonseca JF, Souza-Fabjan JMG, Carvalheira LR, Fernandes DA, Brandao FZ. Efficiency of different hormonal treatments for estrus synchronization in tropical Santa Inês sheep. Trop Anim Health Prod 2016;48:545–51.
[21] Fleiss JL. The design and analysis of clinical experiment. New York: John Wiley Sons; 1986.
[22] Landin JR, Koch GG. The measurement of observer agreement for categorical data. Biometrics 1977;33:159–74.
[23] Jones AK, Reed SA. Benefits of ultrasound scanning during gestation in the small ruminant. Small Rumin Res 2017;149:163–71.
[24] Ishwar AK. Pregnancy diagnosis in sheep and goats: a review. Small Rumin Res 1995;17:37–44.
[25] Petridis IG, Barbagianni MS, Ioannidi KS, Samaras E, Fthenakis GC, Vloumidi EI. Doppler ultrasonographic examination in sheep. Small Rumin Res 2017;152:23–32.
[26] Fricke PM, Lamb GC. Potential applications and pitfalls of reproductive ultrasonography in bovine practice. Vet Clin North Am Food Anim Pract 2005;21:419–36.
[27] Zarco L, Stabenfeldt GH, Quirke JF, Kindahl H, Bradford GE. Release of prostaglandin F-2α and the timing of events associated with luteolysis in ewes with oestrous cycles of different lengths. J Reprod Fert 1988;83:517–26.
[28] Fonseca JF, Mafilli VV, Santos ADF, Fürst R, Prosperi CP, Rovay H, et al. Effects of prostaglandina administration 10 days apart on reproductive parameters of cyclic dairy nulliparous goats. Arq Bras Med Vet Zootec 2012;64:349–58.
[29] Diskin MG, Morris DG. Embryonic and early foetal losses in cattle and other ruminants. Reprod Dom Anim 2008;43:261–8.
[30] Doney JM, Smith WF, Gunn RG. Effects of post-mating environmental stress or administration of ACTH on early embryonic loss in sheep. J Agri Sci 1976;87:133–6.
[31] Sasa A, Teston DC, Rodrigues PA, Coelho LA, Schalch E. Plasma concentrations of progesterone in hair and wool ewe lambs from april to november, in São Paulo state. Braz J Anim Sci 2002;31:1150–6.
[32] Bathaei S. Breeding season and oestrous activity of Iranian fat-tailed Mehraban ewes and ewe lambs. Small Rumin Res 1996;22:13–23.
[33] Farin PW, Britt JH, Shaw DW, Slenning BD. Agreement among evaluators of bovine embryos produced in vivo or in vitro. Theriogenology 1995;44:339–49.
[34] Ginther OJ. Ultrasonic imaging and animal reproduction: color-doppler ultrasonography. Book 4. Equiservice Publishings; 2007.
[35] Arashiro EKN, Palhão MP, Santos JRL, Fontes RC, Siqueira LGB, Henry M, et al. Three-dimensional modeling of color Doppler images: a new approach to study follicular vascularization in cattle. Anim Reprod 2013;10:662–9.
[36] Ginther OJ, Utt MD. Doppler ultrasound in equine reproduction: principles, techniques, and potential. J Equine Vet Sci 2004;24:516–26.
[37] Ginther OJ, Gastal EL, Gastal MO, Utt MD, Beg MA. Luteal blood flow and progesterone production in mares. Anim Reprod Sci 2007;99:213–20.
[38] Herzog K, Bollwein H. Application of doppler ultrasonography in cattle reproduction. Reprod Dom Anim 2007;42:51–8.
[39] Balaro MFA, Santos AS, Moura LFGM, Fonseca JF, Brandao FZ. Luteal dynamics and functionality assessement in dairy goats by luteal blood flow, luteal biometry, and hormonal assay. Theriogenology 2017;95:118–26.