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ORIGINAL ARTICLE

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Assessing the usefulness of B-mode and colour Doppler sonography, and measurements of circulating progesterone concentrations for determining ovarian responses in superovulated ewes

MEF Oliveira[1] | IF Ribeiro[1] | MGK Rodriguez[1] | GS Maciel[1] | JF Fonseca[2] | FZ Brandão[3] | PM Bartlewski[4]

[1]Department of Preventative Veterinary Medicine and Animal Reproduction, School of Agricultural and Veterinary Sciences, São Paulo State University, Jaboticabal, Brazil

[2]Brazilian Agricultural Research Corporation-Embrapa Goats and Sheep, Coronel Pacheco, Brazil

[3]Faculty of Veterinary Medicine, Fluminense Federal University, Niterói, Brazil

[4]Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, ON, Canada

Correspondence
Maria Emilia Franco Oliveira, Department of Preventative Veterinary Medicine and Animal Reproduction, College of Agricultural and Veterinary Sciences, São Paulo State University, Jaboticabal, Brazil.
Email: m.emiliafraoli@fcav.unesp.br

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Contents

The main goal of this study was to assess the usefulness of two imaging modalities, namely the B-mode and colour Doppler sonography, and serum progesterone (P_4) concentrations for determining the ovarian response in superovulated ewes. Twenty-four sexually mature Santa Inês ewes underwent the superovulatory treatment consisting of eight injections of porcine FSH (total dose of 200 or 133 or 100 mg; $n = 8$ ewes/total dose) given at 12-hr intervals and initiated 48 hr before CIDR® (Pfizer Inc., Auckland, New Zealand) removal. Six days after natural mating, the ovaries of all donor ewes were visualized and examined with transrectal ultrasonography and then with videolaparoscopy to identify and enumerate corpora lutea (CL) and luteinized unovulated follicles (LUFs). Jugular blood samples were collected just prior to ovarian examinations. The total number of CL ($r = .78$ and 0.83, $p < .0001$) and LUFs ($r = .74$ and 0.90, $p < .0001$) enumerated using the B-mode and colour Doppler ultrasonographic technique, respectively, were correlated with that ascertained by videolaparoscopy. Circulating concentrations of P_4 were related directly to the number of healthy CL ($r = .73$, $p = .0002$) and inversely to the number of prematurely regressing CL ($r = -.46$, $p = .03$), but the accuracy of predicting the number of short-lived CL with serum P_4 concentrations was very poor. The present results indicate that ultrasonographic imaging and serum P_4 measurements on the day of embryo recovery are useful indicators of total/normal CL numbers and both ultrasonographic techniques can be used to quantify LUFs in superovulated ewes.

1 | INTRODUCTION

The multiple ovulation and embryo transfer (MOET) is a reproductive biotechnology facilitating genetic improvement in livestock species, whose commercial application has increased significantly in recent years (Bartlewski et al., 2016; Oliveira et al., 2012). The high variability and unpredictability of responses to hormonal ovarian stimulation is notably the major challenge preventing further increase in MOET efficiency and its more widespread use in sheep (Menchaca, Vilariño, Crispo, Castro, & Rubianes, 2010; Oliveira, 2011).

At present, the collection of embryos from superovulated ewes is typically accomplished with laparotomy. Although this method offers the highest embryo recovery rates and is less time consuming than laparoscopic embryo flushing (Fonseca et al., 2016), it nonetheless remains an invasive and traumatic procedure. Due to ethical considerations, possibility of post-operative complications and relatively high cost, laparotomy should only be performed when the donor female responded well to the superovulatory treatment (Fonseca, Oliveira, & Viana, 2011). Thus, it is of paramount importance to accurately determine superovulatory responses in individual donor ewes before attempting surgical embryo recovery. The suitability of serum progesterone (P_4) measurements for determining ovarian responses in superovulated sheep has already been studied, but although this method can be used to identify ewes with a high superovulatory response, it is not sensitive enough to predict the exact number of luteal structures (Amiridis et al., 2002). To the best of the authors' knowledge, there has been no study on the relationships between circulating P_4 concentrations and the occurrence of short-lived corpora lutea (CL) or luteinized unovulated follicles (LUF) in superovulated ewes; such associations have only been documented in anoestrous ewes induced to ovulate with gonadotropin-releasing hormone (GnRH; Bartlewski et al., 2001).

Ovarian structures can be visualized and enumerated using exploratory laparotomy, laparoscopy or grey-scale (B-mode) ultrasonographic imaging but the accurate quantification of CL is still only possible with the first two techniques (Oliveira, 2011). Recently, colour Doppler sonography has been employed in cows (Matsui & Miyamoto, 2009) and horses (Witt et al., 2012) as a method to predict the ovarian response after hormonal ovarian superstimulation. Colour Doppler sonography provides a reliable and rapid means of detecting luteal vasculature, which helps delineate individual luteal structures. Similar studies are lacking in small ruminants (Bartlewski et al., 2016; Oliveira et al., 2014).

The main objective of this study was to assess the usefulness of B-mode and colour Doppler sonography for enumerating luteal structures in superovulated ewes. Videolaparoscopic detection and enumeration of luteal structures on the day of embryo recovery were used as a "golden standard" to which ultrasonographic results were compared. We hypothesized that the colour Doppler mode would be associated with the greater accuracy and precision in quantifying various luteal structures as compared with the B-mode scan. In addition, serum P_4 concentrations were examined for correlations with the numbers of detected luteal structures.

Highlights

- The utility of B-mode and color Doppler ultrasonography for detecting corpora lutea (CL) and luteinized unovulated follicles (LUFs) in superovulated ewes was tested.
- Color Doppler technique increased the accuracy of CL enumeration.
- Both methods permitted accurate quantification of LUFs but failed to detect prematurely regressing CL.
- Serum progesterone concentrations were strongly and positively correlated with the number of normal CL.
- More research is needed to improve non-invasive detection and enumeration of inadequate CL in superovulated ewes.

2 | MATERIAL AND METHODS

2.1 | Location, animals and experimental procedures

All experimental procedures were compliant with the guidelines on the ethics and animal welfare, and had been approved by the animal care committee of the College of Agricultural and Veterinary Sciences (FCAV), São Paulo State University "Júlio de Mesquita Filho" (protocol no. 12062-14). This study was conducted in Jaboticabal, SP, Brazil (latitude: 21°15′18″S, longitude 48°19′19″W) from July to October (period of lengthening day lengths) and it utilized 24 sexually mature Santa Inês ewes, 2–3 years old, weighing 35–45 kg and kept under intensive management system with unlimited access to mineral salt licks, water and corn silage, and balanced feed rations (~200 g/animal/day). During the period of lengthening day lengths in southern Brazil, approximately 55% of Santa Inês ewes exhibit recurrent ovulatory cycles (Oliveira, Ayres, Oliveira, Barros, et al., 2016; Oliveira, Ayres, Oliveira, Oba, et al., 2016).

On Day 0 (random day of the oestrous cycle or anovulatory period), all females were fitted with an intravaginal progesterone-releasing device (Eazi-Breed™ CIDR®; Pfizer Inc., Auckland, New Zealand), which was left in place until Day 8. An i.m injection of 37.5 μg of PGF2α analogue (d-cloprostenol; Sincrocio®, Ouro Fino, Brazil) was given at the time of insertion and removal of CIDR devices (Days 0 and 8, respectively). The superovulatory treatment wherein donor ewes received different total doses of exogenous porcine FSH (Group 1: 200 mg, Group 2: 133 mg and Group 3: 100 mg of pFSH i.m., Folltropin®-V; Bioniche Animal Health, Belleville, ON, Canada; $n = 8$ ewes/total superovulatory dose) started 48 hr before the CIDR removal (Day 6). The total doses were administered in eight consecutive applications at 12-hr intervals (20%, 20%, 15%, 15%, 10%, 10%, 5% and 5% of the total pFSH dose; Figure 1). On Day 6 (1st injection of Folltropin®-V) all ewes also received an i.m. injection of 300 IU of equine chorionic gonadotropin (eCG; Novormon®, Syntex, Buenos Aires, Argentina). Fertile rams (introduced 3 days after CIDR withdrawal) were used for oestrous detection and mating (ram to ewes ratio of 1:5).

2.2 | Ultrasonographic techniques and videolaparoscopy

On the day of embryo recovery (6 days after the onset of behavioural oestrus and the beginning of breeding), all animals underwent transrectal ovarian ultrasonography using the colour Doppler and B-mode portable scanner (MyLab VET 30; Esaote, Italy) equipped with a stiffened, variable frequency (6–8 MHz) linear-array transducer. Food and water were withheld 24 hr before ultrasonographic/laparoscopic examinations. All ultrasonographic

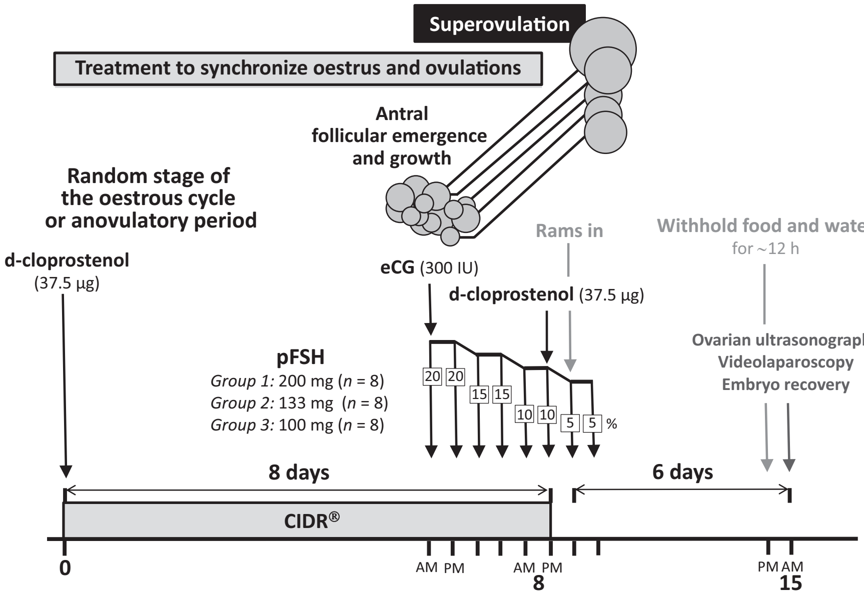

FIGURE 1 A diagram of the experimental protocol including the timing of the ovulation induction protocol, superovulatory procedures and videolaparoscopic/ultrasonographic examinations of ovaries in Santa Inês ewes superovulated in the multiple pFSH regimen using different total doses of Folltropin®-V

examinations were completed by one experienced operator. The ewes were restrained in a standing position and the abdominal wall was compressed to facilitate the visualization of the ovaries. Prior to ultrasonographic examinations, faeces were removed, and the rectum was lubricated with hydrosoluble gel. Videos of the B-mode and colour Doppler ovarian scans were recorded for the identification of the luteal structures; individuals performing those analyses were unaware of the results of laparoscopic examinations. A series of still images of the ovaries (Figure 2) have been captured during each examination. All ovarian images were recorded at constant settings for overall gain and time gain compensation (TGC) and colour Doppler: B-mode-overall gain of 64% of maximum and focal points in the line of view of the ovaries, Doppler sampling frequency (PRF) of 1.4 kHz and colour gain equal to 70% of maximum value or just below the background noise level recorded in a standing, motionless animal. The MyLab Vet 30 cineloop (sequential image storage and review option) spans approximately 12 s during which time 290 frames are saved. To identify and enumerate luteal structures, approximately 30 frames per ovary were analyzed. A typical ovary of the superovulated ewe is approximately 3–4 cm (length) × 2–2.5 cm (width); therefore, the two consecutive frames captured ovarian cross sections separated by ~1.5 mm, which permits the retrospective detection of intraovarian structures ≥2 mm in diameter (Jaiswal, Singh, & Adams, 2004; Schwarz, Murawski, Wierzchoś, & Bartlewski, 2013).

Immediately prior to the laparoscopic procedure, the ewes received i.m. injections of 0.15 mg/kg of 2% xylazine hydrochloride (Rompun®; Bayer HealthCare, São Paulo, Brazil) and of 0.07 mg/kg of acepromazine (Acepran®; Vetnil, Itupeva, Brazil). Epidural anaesthesia was induced with 0.02 mg/kg of lidocaine hydrochloride (Lidovet®; Bravet, Rio de Janeiro, Brazil). Animals were placed on a surgical stretcher at the Trendelenburg position. After disinfecting the skin and applying local anaesthetic (2 ml of lidocaine hydrochloride) at the puncture sites, the trocars were used to access the abdominal cavity and Babcock forceps to gently move and rotate the ovaries. The following three types of luteal structures were enumerated during examinations using the 0° and 7-mm laparoscope (H. Strattner & Cia Ltd.; São Paulo, Brazil) and a video endoscope (Endoflator, H. Strattner & Cia Ltd., São Paulo, Brazil): normal CL (reddish/pinkish luteal structures distinctly protruding above the surface of the ovary; Bartlewski et al., 2017); prematurely regressing CL (≤5 mm in diameter, grossly pale, with little or no protrusion above the surface of the ovary; Rubianes, Ungerfeld, & Ibarra, 1996; Gusmão, Biscarde, & Kiya, 2013); and luteinized unovulatory follicles (luteal structures ≥5 mm and lacking ovulatory stigmata; Bartlewski et al., 2017).

2.3 | Hormone assays

A single jugular blood sample per ewe was collected immediately before each ultrasonographic examination to determine serum concentrations of progesterone (P_4). Blood samples (10 ml) were drawn into evacuated blood collection tubes without anti-coagulants (Becton Dickinson Diagnostics; São Paulo, Brazil). All samples were then centrifuged at 3,000 *g* for 15 min and sera were separated into aliquots properly marked and stored at −20°C until assay at a later date. Progesterone concentrations were measured using a commercial radioimmunoassay kit (Immunotech; Beckman Coulter, Villepinte, France), according to the manufacturer's specifications. The assay sensitivity was 0.1 ng/ml and the range of standards was from 0.1 to 80 ng/ml. All serum samples were analyzed in a single assay with the 18% coefficient of variation.

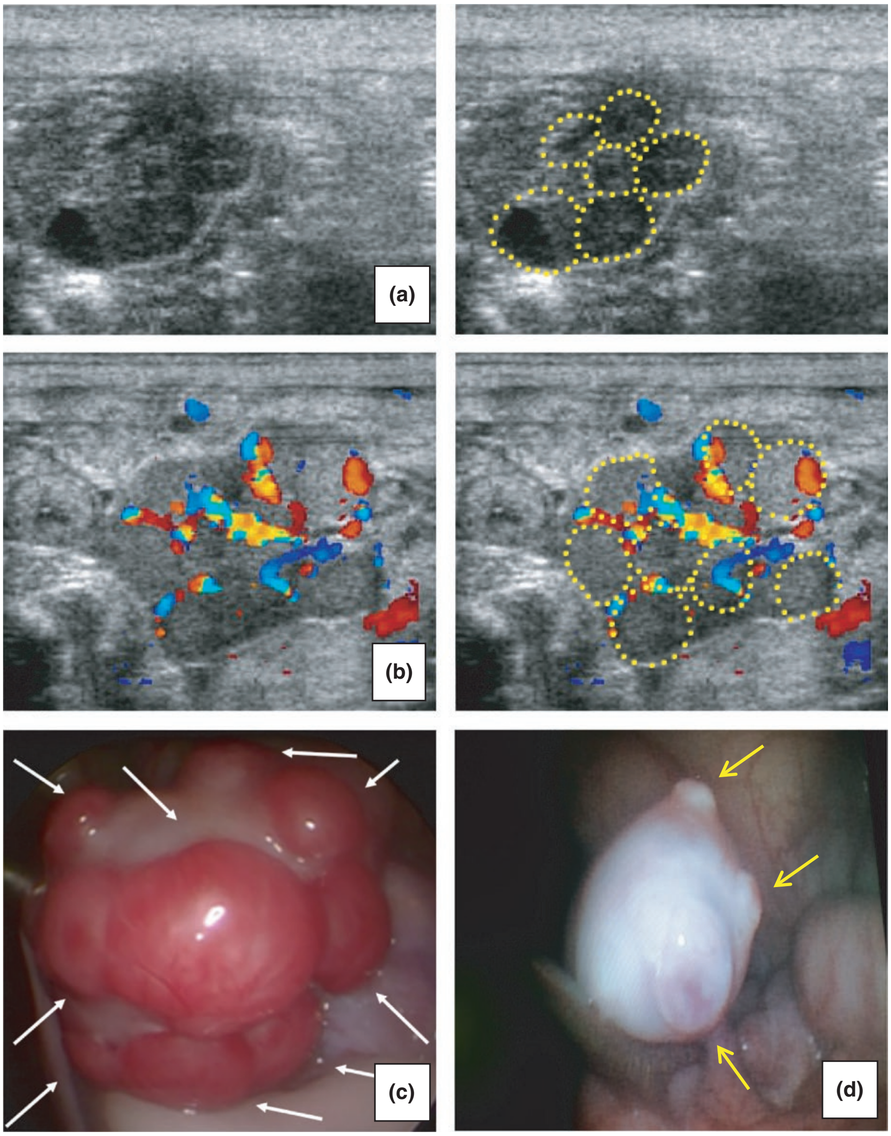

FIGURE 2 Pictures of ovaries in superovulated Santa Inês ewes obtained on the day of embryo collection with B-mode ultrasonography (a), colour Doppler ultrasonography (b) and videolaparoscopy (c and d). Yellow dashed lines indicate the boundaries of corpora lutea (CL) detected in serial ultrasonographic images. Corpora lutea with reddish colouration and protrusion from the surface of the ovary (white arrows, c) are regarded as functional (normal), whereas pale CL (yellow arrows) recorded during laparoscopic examinations were classified as prematurely regressing

2.4 | Statistical analyses

Statistical analyses were performed using the SAS® statistical software (SAS Institute Inc., Cary, NC, USA). Differences between the three treatment groups were determined by one-way analysis of variance (ANOVA). Whenever necessary, the data were transformed by $\log_n$ prior to the analysis; only the total number of CL detected with laparoscopy as well as per cent errors and accuracies of enumerating ovarian structures showed normal distribution (Shapiro–Wilk test). Correlations between ovarian responses (number of CL and unovulated luteinized follicles-LUFs) determined by each technique (B-mode and colour Doppler mode ultrasonography, serum progesterone (P_4) concentrations and videolaparoscopy) and between the numbers of prematurely regressed or normal CL and serum P_4 concentrations were determined using Spearman correlation tests. The accuracy and per cent error of the two ultrasonographic methods to determine the numbers of luteal structures were calculated for individual animals using the videolaproscopic results as the gold standard. The number of CL and LUFs was predicted using a simple linear regression, with serum P_4 concentrations the independent (input) variable. Statistical differences with $p < .05$ were considered significant. Numerical results are expressed as mean ± *SEM*.

3 | RESULTS AND DISCUSSION

All ewes responded to superovulatory treatments and had four or more ovulations/CL; however, a wide individual variation in the superovulatory response was observed (minimum 4 and maximum 24 CL per ewe). Fourteen of 24 ewes had prematurely regressing CL (1–22/ewe) and LUFs were observed in 20/24 ewes (1–5/ewe). Three ewes failed to produce healthy CL and had prematurely regressing CL only. This range of ovulatory responses was not due to the total pFSH dose used (100, 133 or 200 mg per ewe over 4 days; Figure 1, Table 1). High variability in superovulatory outcomes is the major drawback of the MOET biotechnology in sheep; it has been attributed to several intrinsic and

TABLE 1 Mean (±*SEM*) numbers of corpora lutea (CL) and luteinized unovulated follicles (LUFs) detected laparoscopically as well as circulation concentrations of progesterone (P_4) in Santa Inês ewes superovulated in decreasing multiple pFSH with different total doses of porcine follicle-stimulating hormone (pFSH; Folltropin®-V; n = 8 ewes per group)

Group (total pFSH dose)	No. of normal CL	No. of regressing CL	No. of LUFs	P_4 concentrations (ng/ml)
Group 1 (200 mg)	8.6 ± 2.8 (0–23)	6.0 ± 2.9 (0–22)	1.2 ± 0.4 (0–3)	7.5 ± 1.7 (0.3–11.7)
Group 2 (133 mg)	8.6 ± 1.3 (3–14)	1.7 ± 1.1 (0–9)	1.7 ± 0.6 (0–5)	5.8 ± 1.6 (0.1–10.6)
Group 3 (100 mg)	11.1 ± 2.9 (0–24)	2.7 ± 1.7 (0–12)	1.1 ± 0.2 (0–2)	11.2 ± 4.4 (0.1–37.6)

Ranges for individual means are given in parentheses.

TABLE 2 Mean (±*SEM*) numbers of corpora lutea (CL) and luteinized unovulated follicles (LUFs) detected with ultrasonography (B-mode and colour Doppler) and videolaparoscopy in superovulated Santa Inês ewes

Technique or variable	Total no. of CL	No. of normal CL	No. of regressing CL	No. of LUFs
Videolaparoscopy	12.5 ± 1.2 (4–24)	9.5 ± 1.4 (4–24)	3.5 ± 1.2 (0–22)	1.4 ± 0.2 (0–5)
B-mode	13.9 ± 1.2 (5–29)	ND	ND	1.3 ± 0.2 (0–4)
Colour Doppler	12.7 ± 1.1 (4–27)	ND	ND	1.2 ± 0.2 (0–4)
P_4 concentrations (ng/ml)[a]	11.9 ± 0.6 (9–21)	9.7 ± 1.1 (5–27)	3.7 ± 0.4 (0–6)	NS

ND: CL status (healthy vs. regressing) was indistinguishable (not determined).
[a]Numbers of CL were predicted using a linear regression, with serum concentration of P_4 as the independent (input) variable. Regression equations were as follows: Total no. of CL = 9.45 + (0.34 × P_4 concentrations); No. of normal CL = 4.83 + (0.60 × P_4 concentrations); and No. of regressing CL = 5.58 − (0.31 × P_4 concentrations). NS: there were no significant correlations between serum P_4 concentrations and the number of LUFs and so no further analyses were performed.

extrinsic factors (Bartlewski et al., 2016). Due to variable superovulatory responses and the fact that embryo collection in sheep is performed predominantly by a surgical technique (Fonseca et al., 2016), the ability to accurately and non-invasively determine the outcome of hormonal superstimulation would be an invaluable asset.

The laparoscopic technique was used in the present study as the gold standard due to its high accuracy in detecting anatomical structures visible on the surface of the ovary (Oliveira, 2011). However, the operator's skill is critical for accurate quantification of endoscopically monitored ovarian structures. In addition, even though the abdominal laparoscopy is considered semi-invasive, it still bears a possibility of post-operative complications. In particular, the movement of instruments should be performed with extreme caution to avoid any injuries of the ovaries and ovarian pedicle; these structures are richly vascularized (Salles & Araújo, 2010) and reproductive tract haemorrhages can cause the formation of adhesions, which may impair gonadal function. Therefore, the adaptation of transrectal ovarian ultrasonography for the assessment of superovulatory responses would be extremely beneficial, especially in the donor females with high zootechnical and commercial value.

Videolaparoscopy also permits the detection of prematurely regressing CL in superovulated ewes. Premature luteolysis of some CL has been considered another major complication during the in vivo embryo production in sheep, especially in subtropical and tropical climates (Oliveira et al., 2009; Stubbings & Walton, 1986). In these regions, premature luteolysis of some CL occurs in up to 75% of superovulated ewes (Lopes Júnior et al., 2006; Oliveira, 2011; Saharrea et al., 1998). It was suggested that a decline in luteal progesterone concentrations associated with the occurrence of short-lived CL could have adverse effects on embryo quality in ewes undergoing ovarian stimulation (Cervantes et al., 2007; Rodriguez, Campanholi, Maciel, & Oliveira, 2015) resulting in low numbers of transferrable quality embryos (Gomes et al., 2014; Salles, Soares, Andrioli, Sobrinho, & Azevedo, 1998). However, there were no correlations between serum progesterone (P_4) concentrations measured from 1 to 7 days after the 3-day pFSH superovulatory regimen and embryo viability rates in anoestrous Rideau Arcott ewes (Fuerst, Bartlewski, & King, 2009). Most of the Santa Inês ewes in this study (58%, 14/24) had at least one inadequate CL and there were 3.5 ± 1.2 regressing CL per ewe, but there were no statistical differences between the ewes with or without regressing CL in the mean number of viable and degenerated embryos or unfertilized eggs recovered after superovulatory treatments (data not shown).

Ovaries could easily be detected with both ultrasonographic techniques in the ewes of the present study (Figure 2, Table 2). Transrectal ovarian ultrasonography in sheep is performed with a transducer that is held and manipulated externally. Reproductive organs can only be identified on the viewing screen of the echo camera as concurrent palpation of the uterus and ovaries is not possible (Ginther, 2014). Another disadvantage of ultrasonography involves the movement of restrained animals and internal organs that hinders the acquisition and proper interpretation of high-quality images (Black, 2017; Ginther, 2014). Moreover, high numbers of intraovarian structures decrease the accuracy of their

TABLE 3 Summary of correlations (correlation coefficients and *p*-values) between different methods of determining the ovarian response (number of corpora lutea-CL and luteinized unovulated luteinized follicles-LUFs) in superovulated Santa Inês ewes

Technique or variable	Videolaparoscopy			
	Total no. of CL (*p*)	No. of normal CL (*p*)	No. of regressing CL (*p*)	No. of LUFs (*p*)
B-mode	0.81 (<.0001)	–	–	0.77 (<.0001)
Colour Doppler	0.90 (<.0001)	–	–	0.91 (<.0001)
P_4 concentrations (ng/ml)	0.48 (.03)	0.73 (.0002)	−0.46 (.03)	−0.20 (.37)

detection and enumeration (Bergeron, Nykamp, Brisson, Madan, & Gartley, 2013; Viñoles, Meikle, & Forsberg, 2004). Probably the most important limitation of the ultrasound imaging is the operators' qualifications to use it for diagnostic and research-allied purposes (Black, 2017).

In spite of the aforementioned limitations and drawbacks of the ultrasonographic technique, there were strong positive correlations among the total numbers of CL and luteinized unovulatory follicles detected with the two ultrasonographic techniques and those identified with ovarian videolaparoscopy (Table 3). From all indications, both methods can be used to estimate the numbers of all luteal structures with sufficient accuracy. It was somewhat easier to delineate the boundaries of each luteal structure in colour Doppler images than in the B-mode scans. The shape of the corpus luteum is irregular as a part of the structure protrudes above the surface of the ovary; therefore, the counts of CL obtained with B-mode ultrasonography can frequently be overestimated. Colour Doppler sonography has effectively been used in reproductive medicine to evaluate vascularization of ovarian structures including CL (Arashiro et al., 2018; Barbosa & Silva, 2012; Devoto et al., 2009; Lüttgenau & Bollwein, 2014; Miró et al., 2015). The application of colour Doppler sonography enhanced the accuracy of CL enumeration (Table 4); even though the difference was only numerical, the per cent error was reduced 2.5-fold with the use of colour Doppler imaging compared with B-mode ultrasonography.

With regards to detection of LUFs, a very high level of accuracy and moderate per cent errors were associated with the application of both ultrasonographic techniques. At the outset of this study, we assumed that the colour Doppler might facilitate the identification of CL with a large central cavity, which may occasionally be confused with luteinized antral follicles. However, cavitated CL in ewes are usually observed only early in the luteal phase and in late dioestrus/pro-oestrus (Bartlewski, Beard, & Rawlings, 1999).

The vascular system cells constitute approximately 50% of all cell types in the luteal gland (Salles & Araújo, 2010) and during luteolysis, there is a sudden decrease in luteal blood flow due to the local release of vasoactive peptides and, consequently, vasoconstriction (Ayres & Mingoti, 2012). Therefore, colour Doppler sonography may potentially be used to monitor changes in the physiological status or health of individual luteal structures, and to detect prematurely regressing CL (Arashiro et al., 2013). However, using the visual assessment of the present ultrasonographic recordings, it was not possible to identify vascular differences between the normal and inadequate CL. This observation warrants further studies involving computer-assisted image analyses of luteal colour Doppler images from superovulated ewes.

Mean serum P_4 concentrations on the day of embryo collection did not vary ($p > .05$) between the ewes receiving different superovulatory doses of Folltropin®-V (Table 1). A number of earlier studies suggested the existence of a relationship between the number of CL and circulating P_4 concentrations in cyclic ewes (Bindon, Chang, & Turner, 1971; Oyedipe, Pathiraja, Gyang, & Edqvist, 1989). There were no correlations between serum P_4 concentration and ovulatory responses in anoestrous Rideau Arcott sheep superovulated in a multiple-dose pFSH regimen (Bartlewski et al., 2016; Fuerst et al., 2009). Similarly, Amiridis et al. (2002) reported that measurements of serum P_4 concentrations in Chios ewes superovulated with ovine FSH could help identify the best responders but they were not indicative of the exact number of resultant CL. Alternatively, Samartzi, Belibasaki, Vainas, and Boscos (1995) observed that a significant positive correlation existed between plasma

TABLE 4 Per cent errors and accuracies (both mean ± *SEM*) of the two ultrasound imaging modalities and serum P_4 concentrations as methods to determine the numbers of different types of luteal structures in individual superovulated Santa Inês ewes

Technique or variable	Total no. of CL		No. of normal CL		No. of regressing CL		No. of LUFs	
	Error (%)	Accuracy (%)	Error (%)	Accuracy (%)	Error (%)	Accuracy (%)	Error (%)	Accuracy (%)
B-mode	18.4 ± 7.4	73.6 ± 6.2*	–	–	–	–	−12.7 ± 6.9	87.3 ± 6.9
Colour Doppler	7.4 ± 5.4	82.3 ± 4.3*	–	–	–	–	−14.3 ± 7.0	85.7 ± 7.0
P_4 concentrations (ng/ml)	19.5 ± 13.1	64.4 ± 5.0*	18.8 ± 15.0	66.4 ± 4.4	74.5 ± 43.3	25.3 ± 9.4	–	–

*$p < .05$.

P_4 concentration on the day of embryo recovery and the number of CL in Chios ewes superovulated with pregnant mare serum gonadotropin (PMSG). Those discrepancies among previous reports might be due to the breed- and season-related influences or exogenous gonadotropins used as well as differences in the prevalence of prematurely regressing CL and/or LUFs in ewes undergoing superovulatory treatments. There has been no earlier study of correlations between serum P_4 concentrations and different types of the luteal structures detected in superovulated sheep. In the present study, serum P_4 concentrations determined on the day of embryo recovery were related directly to the total number of CL ($r = .48$, $p = .03$) and numbers of normal CL ($r = .73$, $p = .0002$), and inversely to the number of prematurely regressing CL ($r = -.46$, $p = .03$; Table 3). Therefore, circulating P_4 concentrations are a good indicator of the ovarian superovulatory response (total number of CL) but especially the number of normal (healthy) CL in pFSH-treated Santa Inês ewes. The accuracy of estimating the total number of CL was, however, less ($p < .05$) with serum P_4 measurements compared with colour Doppler sonography, but not than that achieved using B-mode scanning (Table 4). Albeit serum P_4 concentrations differed significantly between ewes with or without regressing CL (4.5 ± 1.1 ng/ml compared with 12.6 ± 3.2 ng/ml), due mainly to low accuracy/relatively high per cent error and high variability in serum P_4 concentrations it was not possible to distinguish the ewes with ovaries bearing ≤10 ($n = 21$) or >10 ($n = 3$) prematurely regressing CL, and any finer divisions were also statistically impossible. Lastly, serum P_4 concentrations are a poor indicator of the numbers of unruptured ovarian antral follicles following the superovulatory pFSH treatment of ewes.

In summary, the present results are supportive of the utility of B-mode and colour Doppler ultrasonography as the practical, non-invasive methods to determine the ovarian response in superovulated ewes, in a commercial setting or reproductive research. The colour Doppler technique appeared to increase the accuracy of CL detection and enumeration. Serum P_4 concentrations are primarily related to the number of fully functional, healthy CL but they are not predictive of the number of prematurely regressing CL or LUFs. More research is needed to identify prematurely regressing CL with the ultrasonographic technology as they could not be distinguished from healthy CL using visual assessment of either B-mode or colour Doppler ultrasonographic images.

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CONFLICTS OF INTEREST

There is no conflict of interests to disclose.

AUTHOR CONTRIBUTIONS

The present experiment was originally designed by M.E.F. Oliveira and J.F. Fonseca. The acquisition, analyses and interpretation of the data were done by all authors, and manuscript preparation was the primary responsibility of M.E.F. Oliveira and P.M. Bartlewski. All authors have read and approved of the submitted and revised versions of the paper.

ORCID

MEF Oliveira http://orcid.org/0000-0002-7730-290X

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