Small Ruminant Research 216 (2022) 106803

Contents lists available at ScienceDirect

Small Ruminant Research

journal homepage: www.elsevier.com/locate/smallrumres

Ovarian assessment for pre-selection of embryo donor ewes

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ARTICLE INFO

Keywords:
Embryos
Santa Inês
Ovarian size
Follicle
Ultrasound

ABSTRACT

The present study aimed to evaluate whether two-dimensional ovarian measurements and the identification of antral follicle populations by ultrasound at different stages of the estrous cycle are related to the superovulatory response and embryo production in ewes. The estrous cycle of 57 Santa Inês ewes was synchronized by administering two doses of 75 µg of prostaglandin (PGF) 10 days apart. After 2.5 (proestrus/estrus), 4.5 (metestrus), and 9.5 (diestrus) days from the last PGF injection, ultrasound examinations were performed to quantify antral follicles $\geq$ 2 mm and ovarian measurements. Subsequently, the number of follicles and ovarian measurements were correlated with the superovulatory response and embryo production of each ewe. The mean follicle population ($\geq$ 2 mm) and ovarian dimensions were similar in all phases of the estrous cycle ($P > 0.05$). Positive correlations were identified between the number of follicles and ovarian dimensions ($P < 0.05$) in different phases of the estrous cycle. Donor ewes with a high number of follicles ($\geq$ 7) in the metestrus phase showed a significant increase in the number of ovulations, total structures, total embryos, and viable embryos ($P < 0.05$). In conclusion, ultrasonographic assessment of the number of follicles $\geq$ 2 mm, performed during metestrus (4.5 days after synchronization pre-treatment), is an easy-to-perform approach and can be used for pre-selection of embryo donor ewes.

1. Introduction

Multiple ovulation and embryo transfer (MOET) technologies have been applied in ewes for embryo conservation in germplasm banks and to increase the progeny of selected ewes. Traditionally, MOET technology involves synchronizing estrus using progesterone/progestin for 12 or 14 days and administering supraphysiological doses of gonadotropins to induce growth and ovulation of many ovarian follicles (Cognie, 1999; Brasil et al., 2014; Khan et al., 2022). After hormonal treatment, embryos are surgically retrieved from the reproductive tract and transferred to receptors or cryopreserved.

The success of a MOET program is determined by intrinsic and extrinsic factors, including season, genetics, age, nutrition, management, stress, type of gonadotropins used, and treatment protocols adopted (Gonzalez-Bulnes et al., 2004). Although there has been greater control over extrinsic factors, ovarian response is still variable, suggesting that intrinsic factors are the main factors responsible for this variability (Bartlewski et al., 2016).

Many studies have shown that the ovarian response is related to the characteristics of the ovary at the onset of superstimulation with exogenous gonadotropins (Rubianes et al., 1995; Gonzalez-Bulnes et al., 2000, 2002; Veiga-Lopez et al., 2005; Mossa et al., 2007; Bartlewski et al., 2008). Most of these studies indicate that the number of small follicles is positively correlated with the ovulation rate and the number of viable embryos (Gonzalez-Bulnes et al., 2000, 2002; Mossa et al., 2007).

In cattle, ovary weight (Murasawa et al., 2005) and volume (de Lima et al., 2020) are correlated with the follicular population. In each follicular wave, there can be a sevenfold variation in the number of small follicles among individuals (Burns et al., 2005). In ewes, high intra-individual repeatability is observed after successive superovulation treatments (Cordeiro et al., 2003; Bruno-Galarraga et al., 2014). This consistency is possibly related to the follicular population intrinsic to each female and, consequently, to follicular development.

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https://doi.org/10.1016/j.smallrumres.2022.106803
Received 25 May 2022; Received in revised form 17 August 2022; Accepted 21 August 2022
Available online 25 August 2022
0921-4488/

In a previous study, it was verified that the dimensions of the ovarian tissue area, assessed by ultrasound, are noninvasive indicators of ovarian follicular population (Bowen et al., 2007) and the potential success of assisted reproductive technologies applied to women (Lass et al., 1997; Syrop et al., 1999). However, to the best of our knowledge, studies of this nature have not yet been carried out on sheep.

The creation of a methodology for pre-selection of embryo donor allows cost reduction of hormonal protocols and avoids unnecessary surgical procedures in non-responsive ewes. Therefore, this study aimed to evaluate whether two-dimensional ovarian measurements and identification of the population of antral follicles by ultrasonography at different stages of the estrous cycle are related to the superovulatory response and embryo production in ewes.

2. Material and methods

2.1. Experimental station and animals

The study was carried out at Embrapa Genetic Resources and Biotechnology located in Brasília (central-west region of Brazil) at 15°88′ latitude S and altitudes ranging from 1050 to 1250 m above sea level. According to the Köppen classification, this region has a rainy tropical climate with dry winters and rainy summers (Alvares et al., 2013). Ethical concerns were addressed in accordance with the local regulations on animal welfare practices.

Fifty-seven sexually mature, clinically healthy Santa Inês ewes, aged 2–5 years and with a body condition score > 2.5 (scale 1–5), were used in this study. All ewes were kept in collective pens and fed Tifton hay and balanced feed (150 g/ewe/day) with access to water and mineral salt ad libitum.

2.2. Experimental procedure

The estrus of all ewes was synchronized by administering two doses of 75 μg D-cloprostenol (Prolise®, Tecnopec, ARSA S.R.L., Argentina) with a 10-day interval between applications (Godfrey et al., 1997). After the last injection of D-cloprostenol (day 0), on days 2.5, 4.5, and 9.5, the time corresponding to proestrus/estrus, metestrus, and diestrus, respectively, ultrasound examinations were performed to evaluate the ovaries and quantify follicles ≥ 2 mm.

The ovary size was calculated using the two-dimensional formula validated for women, as described by Frattarelli et al. (2002), through the largest diameters of the sagittal plane of each ovary [(D1 + D2) / 2], and the average for each ewe was recorded (Fig. 1). The animals in this experiment were subjected to a superovulation procedure 10 d after the last ultrasonographic evaluation of the ovaries. The number of follicles and the two-dimensional size of the ovary in the proestrus/estrus, metestrus, and diestrus phases were evaluated for their correlation with superovulation and embryo yield.

2.3. Ultrasound evaluation

Ovaries were assessed by an experienced operator using high-resolution real-time B-mode ultrasound equipment (DP-2200Vet; Shenzhen Mindray Bio-Medical Electronics Co. Ltd., Nanshan, Shenzhen, PR China) equipped with a multifrequency linear transducer at 7.5 MHz frequency. The ewes were subjected to an ultrasound examination at the station position in a containment trunk. This technique has been previously validated for monitoring ovarian follicular dynamics and detecting corpora lutea (CL) in sheep (Viñoles et al., 2004).

2.4. Superovulation and embryo collection protocol

Sheep were treated with CIDR (EAZI-BREEDTM, CIDR®, New Zealand) inserts for 7 days (4 or 6 sheep/day) to synchronize the estrous cycle. On day 4, superovulation was induced using 133 mg of pFSH IM (Folltropin®-V, Tecnopec, AHC Inc., Bioniche, Canada) administered in eight decreasing doses with a 12-h interval (20% × 2, 15% × 2, 10% × 2%, and 5% × 2), starting on the morning of day 4 and ending 12 h after CIDR removal. A dose of PGF2α (250 μg of cloprostenol; Sincrocio®, Ouro Fino Animal Health LTDA, Brazil) and GnRH (25 μg of gonadorelin acetate; Gestran Plus, Tecnopec, ARSA SRL, Argentina) was administered IM together with the 5th and 8th pFSH injection, respectively.

All females were inseminated at 36 and 48 h after CIDR removal using fresh semen (0.5 mL), previously collected from three rams, pooled, and kept in a water bath at 33 °C. On the fifth day after the second AI, the number of ovulations was verified using a laparoscopic procedure. Ewes with > 2 CL were considered responsive donors.

Superovulated donors were anesthetized with xylazine (0.10 mg/kg IM; Rompun®, Bayer, Brazil) and ketamine hydrochloride (3.5 mg/kg IV; ketamine, Agener, Brazil). In addition, local anesthesia (10 mL of lidocaine; Lidovet®, Bravet, Brazil) was administered in the region of the surgical incision. Oocytes/embryos were surgically collected after ventral laparotomy through a paramedian incision (5 cm long) cranial to the udder to access the reproductive tract. Each uterine horn was washed with 60 mL of embryo recovery medium (DPBS; Cultilab, Brazil), prewarmed to 38 °C, and supplemented with 1% fetal bovine serum (Cultilab, Brazil). Embryos were retrieved in a Petri dish, kept in holding medium (Holding Plus, 0.4% BSA, Embriocare, Cultilab, Brazil), and examined under a stereomicroscope (Olympus SZ; Olympus Optical Co., Ltd., Tokyo, Japan) at 20–40 × magnification, following the criteria of the International Embryo Transfer Society (Da Stringfellow and Givens, 2010). Embryos that developed to blastocyst or morula stage were classified as follows: grade 1 (excellent or good), 2 (good/fair), 3

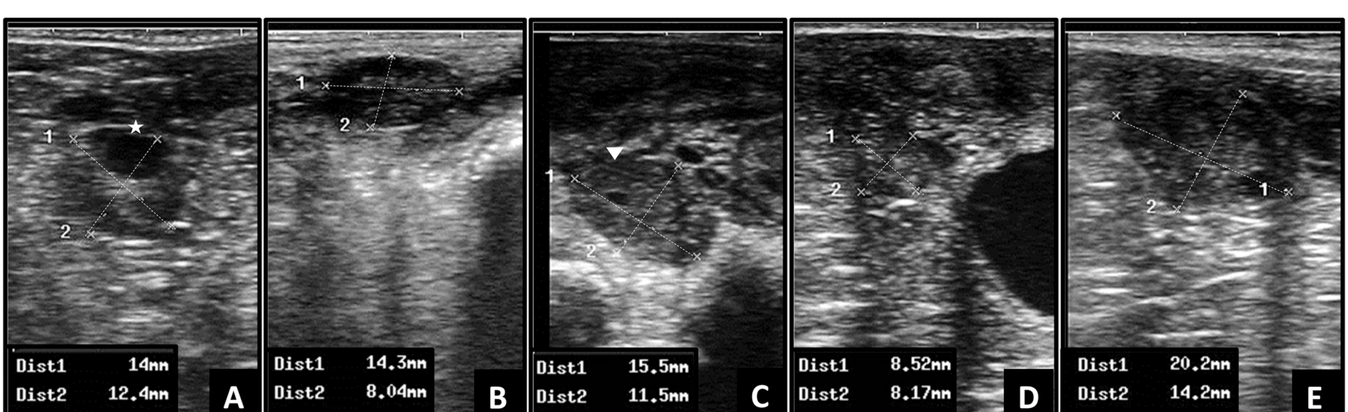

Fig. 1. Ultrasonographic image of the two largest diameters of the sagittal plane of the ovary at different stages of the estrous cycle. Proestrus/estrus (A) with the presence of a large follicle (star); metestrus (B); diestrus (C) with the presence of a corpus luteum (arrow); small ovary (D); and large ovary (E).

(poor), and 4 (dead or degenerate). Embryos classified as grades 1–3 were considered viable.

2.5. Statistical analysis

Statistical analyses were performed using the SAS University Edition program (SAS Institute Inc., Cary, NC, USA), with differences considered significant at $P \leq 0.05$. The type of distribution of variables was evaluated using the Kolmogorov-Smirnov goodness-of-fit test. We classified the ewes into two groups based on the mean number of follicles, one with high follicular number (≥ 7) and another with low follicular number (< 7). The mean number of follicles was calculated after evaluating the follicular population in the three different phases of the estrous cycle. The number of follicles and diameter of the ovary in the three phases were analyzed using the mixed generalized linear model (GLIMMIX procedure), with exponential distribution of variables and least squares means adjusted for multiple comparisons using the Royen-Tukey-Kramer test. Spearman's correlations were performed between the ovarian evaluation parameters and the superovulatory response and the embryo productivity of the donors. Subsequently, the superovulatory response and embryonic yield of ewes with low or high follicular population in the three phases of the estrous cycle were analyzed using Mann-Whitney test.

3. Results

The proestrus/estrus, metestrus, and diestrus phases were characterized on days 2.5, 4.5, and 9.5 after the second PGF application. In the proestrus/estrus phase, 84% (48/57) of ewes had at least one follicle ≥ 4.5 mm. In the metestrus phase, only 10% (6/57) of the animals had an identifiable CL, while in the diestrus phase, 89% (51/57) of the ewes had a well-developed CL.

The mean follicle population (≥ 2 mm) and the dimensions of the ovaries were similar between all phases evaluated, with the overall mean population being 6.7 ± 3.2 (range: 0 – 15 follicles; $P > 0.05$) and 12.3 ± 1.7 mm in diameter (range: 8.72 – 18.8 mm; $P > 0.05$), respectively. The number of follicles identified in proestrus/estrus was positively correlated ($P < 0.05$) with the number of follicles present in metestrus and diestrus, as well as with the ovarian dimensions measured in proestrus/estrus, and diestrus (Table 1). In metestrus and diestrus, the number of follicles showed a positive correlation ($P < 0.01$) with the ovarian dimensions evaluated in different phases of the estrous cycle (Table 1). The ovarian dimensions were positively correlated ($P < 0.001$) with each other in the three different phases of the estrous cycle (Table 1).

The correlations of the number of follicles and ovarian dimensions with the superovulatory response of donor ewes are shown in Table 2. The number of follicles in diestrus, and ovarian dimensions were not correlated with the response of the donor ewes subjected to superovulation ($P > 0.05$). However, all parameters related to ovulatory response and embryo yield were positively correlated with the number of follicles in proestrus/estrus, and metestrus ($P < 0.05$), except between the number of follicles in proestrus/estrus and the total number of ovulations.

The effect of the number of ovarian follicles identified in different phases of the estrous cycle on ovarian response and embryo production in ewes subjected to superovulation is shown in Table 3. The follicular population identified in the diestrus phase was not associated with superovulatory response ($P > 0.05$). In contrast, ewes with a high number of follicles (≥ 7) in the metestrus phase showed a significant increase in the number of ovulations, total structures, total embryos, and viable embryos ($P < 0.05$). In addition, in ewes that had a high number of follicles (≥ 7) in the proestrus/estrus phase, a significant increase in the total number of embryos produced was observed ($P < 0.05$).

4. Discussion

The results of this study support the idea that a simple ultrasound analysis can be used to predict the responsiveness of embryo donor ewes to superovulatory treatments. Although the ovarian diameter was not an indicator, quantification of the follicular population ≥ 2 mm can be used before superstimulation with FSH to select females that are more responsive to the protocol.

In the present study, there was high inter-individual variability in the number of follicles (range: 0 – 15), as reported by other authors (Mossa et al., 2007; Bruno-Galarraga et al., 2014). The reason for this variability remains unknown; however, genetic factors may be the main reason for

Table 1
Correlation (r-value) between ovarian parameters, evaluated in different phases of the estrous cycle, used for pre-selection of embryo donor ewes.

	FP/E	FM	FD	DP/E	DM
FM	0.37 * *				
FD	0.47 * **	0.23			
DP/E	0.29 *	0.37 * *	0.43 * **		
DM	0.20	0.39 * *	0.42 * *	0.76 * **	
DD	0.30 *	0.35 * *	0.50 * **	0.72 * **	0.76 * **

Asterisks indicate significant correlations: *P = 0.01 – 0.05; * *P = 0.001 – 0.009; * **P < 0.001.
Number of follicles in proestrus/estrus (FP/E), metestrus (FM), and diestrus (FD).
Ovarian dimensions during proestrus/estrus (DP/E), metestrus (DM), and diestrus (DD).

Table 2
Correlation (r-value) between the number of follicles or ovarian dimensions at different stages of the estrous cycle and the superovulatory response and embryo yield of donor ewes.

Donor ewes' response	Number of follicles[1]			Ovarian Dimension[2]		
	FP/E	FM	FD	DP/E	DM	DD
Total ovulations	0.22	0.28 *	0.08	0.25	0.05	0.07
Total structures	0.34 *	0.39 * *	0.15	0.24	0.08	0.11
Total embryos	0.40 * *	0.46 * **	0.10	0.21	0.11	0.10
Viable embryos	0.36 * *	0.40 * *	0.11	0.18	0.06	0.05

Asterisks indicate significant correlations: *P = 0.01 – 0.05; * *P = 0.001 – 0.009; * **P < 0.001.
Number of follicles in proestrus/estrus (FP/E), metestrus (FM), and diestrus (FD).
Ovarian dimensions during proestrus/estrus (DP/E), metestrus (DM), and diestrus (DD).

Table 3
Ovarian response and embryo production (mean ± standard deviation) in ewes with a high (≥ 7) or low (< 7) number of follicles, in different phases of the estrous cycle, submitted to the superovulation protocol.

Number of follicles		Ovulations	Structures	Embryos	Viable embryos
Proestrus/ estrus	n				
High	30	9.5 ± 7.0	6.9 ± 4.9	6.2 ± 4.8^a	4.9 ± 4.4
Low	27	7.6 ± 5.2	4.9 ± 5.1	3.6 ± 4.1^b	3.0 ± 4.1
Metestrus	n				
High	33	10.9 ± 7.2^c	8.2 ± 4.8^c	7.0 ± 4.6^c	5.4 ± 4.3^c
Low	24	6.1 ± 4.4^d	3.4 ± 4.3^d	2.6 ± 3.8^d	2.1 ± 3.8^d
Diestrus	n				
High	25	9.8 ± 7.9	7.5 ± 5.5	6.3 ± 5.5	4.8 ± 4.8
Low	32	8.2 ± 5.3	6.3 ± 4.5	4.3 ± 4.0	3.4 ± 4.0

Different letters in the columns indicate significant differences at each stage of the estrous cycle (^{a,b} $P < 0.05$; ^{c,d} $P < 0.01$).

the variation among individuals (Bindon et al., 1986; Monget et al., 2002).

High intra-individual repeatability in the number of follicles occurs among follicular waves in cattle (Burns et al., 2005; Morotti et al., 2017). In sheep, the number of small follicles (1 – 3 mm in diameter) is constant throughout the estrous cycle (Duggavathi et al., 2003). In the present study, although positive correlations were observed in the number of follicles among some of the studied phases, these correlations were low (Table 1). According to Scaramuzzi et al. (2011), the population of gonadotropin-responsive follicles shows little numerical change throughout the cycle, unlike gonadotropin-dependent follicles that grow in a wave-like pattern. Based on these findings, to obtain greater repeatability among the studied phases, the quantification of all follicles ≥ 1 mm would be necessary; however, because of the complexity of analyzing such follicles, this method becomes impractical to perform under field conditions in sheep.

The number of small follicles assessed at the first administration of FSH has been positively correlated with the ovulation rate and the number of viable embryos in many studies (Gonzalez-Bulnes et al., 2000, 2002; Mossa et al., 2007). However, other authors have not verified this correlation (Bruno-Galarraga et al., 2014; Brasil et al., 2021). In this study, the superovulatory response and embryo production were positively correlated with the number of follicles ≥ 2 mm in metestrus. No correlation with total ovulations was observed in proestrus/estrus, and no correlation was observed with any of the analyzed variables in diestrus. These results indicate that the evaluation of the population of follicles ≥ 2 mm is only valuable as a tool for pre-selection of donors in some phases of the estrous cycle. When the animals were divided into high and low follicular populations, the results of superovulatory response and embryo yield confirmed the correlations observed in each studied phase (Table 3), indicating that the quantification of follicles in metestrus is the best phase for donor selection.

The proestrus/estrus, and metestrus phases corresponded to days 2.5 and 4.5 after the second application of PGF, respectively. Within this interval, ovulation occurs in protocols with prostaglandins (Fierro et al., 2013; Olivera-Muzante et al., 2020), and on the day of ovulation, the emergence of the first follicular wave is observed (Bartlewski et al., 1999; Toosi et al., 2010). Therefore, it seems that the evaluation of the number of follicles ≥ 2 mm has a greater correlation with the superovulatory and embryo yield parameters when performed closer to the emergence of the follicular wave. A possible alternative to quantify follicles ≥ 2 mm at a more predictable time of follicular wave emergence would be the use of the progesterone protocol associated with the application of 0.5 mg of 17β-estradiol (17β-E), which allows the recruitment of the synchronized form wave 2.8 days after 17β-E administration (Brasil et al., 2021).

Ovarian diameter had high inter-individual variability. To our knowledge, this is the first report in sheep that attempts to demonstrate the relationship between ovarian diameter, as measured by ultrasound, and ovarian responsiveness to gonadotropins. We hypothesized that ultrasound assessments of ovarian dimensions would be a good indicator for predicting ewes' responsiveness to superstimulation treatments, although this was not confirmed by the data obtained in this study (Table 2).

The ovarian dimensions were positively correlated with each other in the three different phases of the estrous cycle. This finding allows us to infer that transrectal ultrasound is a reliable method for assessing the dimensions of the ovary in different phases of the estrous cycle. However, it is important to emphasize that in diestrus, the size of the corpus luteum can interfere with the measurement of the ovary, especially when it is very protruding. Therefore, to obtain a more representative image of the ovary, the ovarian parenchyma must be delimited with the least possible interference from the corpus luteum.

In the present study, ovary size was not correlated with the number of ovulations or embryo yield. However, despite being low, there was a positive correlation between the number of follicles and the size of the ovary among almost all phases studied (range 0.29 – 0.50). It is reasonable to believe that a larger number of follicles requires a larger ovary; however, a large ovary does not necessarily guarantee a larger number of follicles. In this case, the ovary may have many areas of cortical tissue devoid of follicles, which is negatively correlated with the number of preantral and antral follicles, as well as being related to a low superovulatory response (Cushman et al., 1999).

Follicular quantification by ultrasound is a simple and promising approach for field selection of embryo donor ewes. In addition, this approach can be associated with other screening methodologies, including hormone measurements (Gonzalez-Bulnes et al., 2002; Fuerst et al., 2009; Lahoz et al., 2014; Pinto et al., 2018) and information from previous superovulation treatments (Bruno-Galarraga et al., 2014; Balaro et al., 2016) to increase the accuracy of pre-selection and avoid unnecessary expenses and surgical procedures.

5. Conclusions

Ultrasonographic assessment of the number of follicles ≥ 2 mm, performed 4.5 days after prostaglandin synchronization pre-treatment, is a simple test that can be used to select embryo donor ewes. However, the ultrasonographic evaluation of ovarian dimensions, although showing high repeatability between evaluations, does not demonstrate efficacy as a method of selecting embryo donor ewes.

Acknowledgements

The authors thank CAPES for the scholarship and EMBRAPA for the use of materials and facilities. We would like to thank Editage (www.editage.com) for English language editing.

List of funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Disclosure statement

Declarations of interest: none.

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