Small Ruminant Research 211 (2022) 106703

Contents lists available at ScienceDirect

Small Ruminant Research

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

Comparison of superovulatory responses to a standardized hormonal superstimulation protocol among three indigenous breeds of sheep in Brazil

Maria Amélia Ferrão Pupin [a], Maria Emilia Franco Oliveira [a,b,*], Gabriel Brun Vergani [a], Monalisa Sousa Dias Lima [c], Kleibe de Moraes Silva [b], Alexandre Weick Uchôa Monteiro [b], Alexandre Floriani Ramos [d], Ribrio Ivan Tavares Pereira Batista [e], Joanna Maria Gonçalves Souza-Fabjan [f], Pawel Mieczyslaw Bartlewski [g], Jeferson Ferreira Fonseca [b]

[a] Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Jaboticabal, SP, Brazil
[b] Embrapa Caprinos e Ovinos, Sobral, CE, Brazil
[c] Universidade Estadual do Ceará, Fortaleza, CE, Brazil
[d] Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF, Brazil
[e] Universidade Federal do Vales dos Jequitinhonha e Mucuri, Teófilo Otoni, MG, Brazil
[f] Universidade Federal Fluminense, Niterói, RJ, Brazil
[g] Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, ON, N1G 2W1 Canada

ARTICLE INFO

Keywords:
Ovarian antral follicles
Corpora lutea
Color Doppler
Ovarian blood perfusion
Superovulation
Ewe

ABSTRACT

This study examined the differences in ovarian responses and in vivo embryo production in three naturalized Brazilian sheep breeds (Morada Nova, Santa Inês, and Somalis Brasileira). Thirty cycling, multiparous ewes (n = 10/breed) received intravaginal devices containing 0.33 g of progesterone (CIDR®) for 9 days and the twice-daily superovulatory treatment with 133 mg of porcine follicle-stimulating hormone (six decreasing doses) started on Day 7 (Day 0 PM = CIDR® insertion). On Days 11 (36 h after CIDR® removal) and 15 (12 h before non-surgical embryo recovery performed seven days after CIDR® removal), all ewes were examined using B-mode and Doppler ultrasonography. The number of medium-sized ovarian follicles (4.0–5.99 mm) on Day 11 was greater (P = 0.003) in Santa Inês and Somalis Brasileira compared with Morada Nova ewes. Small antral follicle numbers (2.0–3.99 mm in diameter) on Day 11 were directly related to ovulatory responses in all three breeds of superovulated ewes. The number of corpora lutea on Day 15 was greater ($P < 0.0001$) in Santa Inês, followed by Somalis Brasileira and Morada Nova ewes; however, the number of viable embryos and viability rate did not differ ($P > 0.05$) among the three genotypes of ewes studied. The total ovarian area (TA), color Doppler area (DA), and DA/TA × 100% increased from Day 11 to Day 15 in all breeds, and they were lowest ($P < 0.001$) in superovulated Morada Nova sheep. Significant positive correlations among ovarian antral follicle numbers in different size classes (Day 11) and ovulatory responses were recorded in all breeds, but associations between the ovarian blood perfusion and superovulatory outcomes were restricted to the Morada Nova and Santa Inês genotypes. In summary, ovarian follicle numbers and blood flow indices differed among the three naturalized Brazilian ewes, but no significant differences were noted in embryo yields and quality following superovulation. Small antral follicle count 36 h after CIDR® treatment was a reliable predictor of impending ovulation rates in all ewes under study.

1. Introduction

Multiple ovulation and embryo transfer (MOET) technique has extensively been studied to improve its efficiency and further accelerate the expansion of flocks and breeding programs in ruminant species (Maciel et al., 2017). Considerable variability in responses obtained with MOET has greatly limited the widespread use of this biotechnology in sheep (Bari et al., 2001; Célia et al., 2012; Pinto et al., 2020). The

* Correspondence to: Via de Acesso Prof. Paulo Donato Castelane, S/N - Vila Industrial, Jaboticabal, SP, 14884-900, Brazil.
E-mail address: mef.oliveira@unesp.br (M.E.F. Oliveira).

https://doi.org/10.1016/j.smallrumres.2022.106703
Received 21 November 2021; Received in revised form 21 March 2022; Accepted 15 April 2022
Available online 19 April 2022

unpredictability of responses has been due to both the intrinsic and extrinsic factors (Bartlewski et al., 2016), and it can result in very successful or near-failure outcomes even when identical superovulatory (SOV) protocols are used (Baldassarre and Karatzas, 2004).

The intrinsic factors that can impinge on superovulatory outcomes in sheep include, but are not limited to, the breed, age, and follicular population present at the beginning of the superovulatory protocol (Bartlewski et al., 2016). Few studies have directly documented differences in ovarian responses and viable embryo yields produced with MOET programs in various genotypes of sheep (Ammoun et al., 2006; Brasil et al., 2018; Rebolledo et al., 2017). For instance, Dorper ewes showed better overall responses (number of corpora lutea) in comparison with Katahdin, Blackbelly, and Pelibuey ewes in Mexico during warm-dry climate (Rebolledo et al., 2017), and Rubia del Molar had a greater number of corpora lutea and oocytes/embryos compared with Manchega and Negra de Colmenar breeds in Spain during the breeding season (Ammoun et al., 2006). Better superovulatory responses were also obtained in Morada Nova compared with Somalis Brasileira ewes in tropical climate (Brasil et al., 2018). Thus, the variability in superovulatory yields among different sheep breeds demonstrated calls for the establishment of protocols optimized for each breed, particularly when this involves endangered populations of animals (Ammoun et al., 2006) or valuable breeds and species. The Morada Nova, Santa Inês, and Somalis Brasileira breeds are native of Brazil and highly prevalent in the northeastern region of the country that has the largest population of small ruminants in Brazil. These three breeds are well adapted to the northeastern semi-arid environment of Brazil, exhibiting great rusticity and aptitude for meat and milk production as well as significant socio-economic importance for small and medium farm operations (Brasil et al., 2018; Rajab et al., 1992).

Another intrinsic factor related to ovarian responses and embryo quality in hormonally superstimulated ewes is ovarian blood perfusion. The usefulness of ovarian color Doppler ultrasonography to predict the superovulatory responses has been tested in sheep; however, its application still progresses rather slowly (Bartlewski, 2019). Studies of antral follicular blood flow in superovulated Santa Inês ewes revealed that color Doppler signal of the follicular wall on the last day of the 4-day superovulatory protocol was directly related to the number of unfertilized oocytes (Oliveira et al., 2014). High-velocity follicular blood flow at various time points during the 4-day superovulatory treatment was also indicative of the efficiency of in vivo embryo production (Oliveira et al., 2017). Elevated ovarian blood flow velocity is associated with the terminal development of ovulatory follicles in women (Lass and Brinsden, 1999), but there has been no ultrasonographic study of the periovulatory changes in ovarian blood flow in superovulated ewes. In general, an increase in ovarian blood perfusion is indicative of the health of intraovarian structures (Clark and Stokes, 2011; Van Blerkom et al., 1997). Thus, we hypothesized that the ovarian blood perfusion rates and flow velocities determined during the terminal stages of ovulatory follicle development would be indicative of superovulatory responses.

The present experiment was undertaken to examine and compare ovarian antral follicle populations during the preovulatory stage of their development as well as ovarian blood perfusion following estrus synchronization and 12 h before non-surgical embryo recovery in the three indigenous genotypes of Brazilian sheep (Morada Nova, Santa Inês and Somalis Brasileira) following an application of the same superovulatory protocol (i.e., the 3-day declining-dose pFSH superovulatory regimen). Quantitative correlations among antral follicle numbers, ovarian blood perfusion data, ovarian responses and embryo yields/quality were also evaluated.

2. Materials and methods

2.1. Location, ethics, and experimental animals

The present study was carried out at the Experimental Campus of Embrapa Goats and Sheep located in Sobral, CE, Brazil (latitude 03º41'10 "S and longitude 40º20'59" W) between October and November. Due to the city's proximity to the equator, annual variations in day length are very small and so the seasonal pattern of reproduction is less pronounced or completely absent in sheep, allowing for ovarian cyclicity to occur throughout the year in many breeds. The Embrapa Dairy Cattle Ethics Committee on the Use of Animals (CEUA/EGL; no. 2512100516) approved all experimental procedures.

Thirty clinically healthy, multiparous ewes of naturalized Brazilian breeds (Morada Nova, Santa Inês and Somalis Brasileira; $n = 10$/breed) were used in this study. The mean (± SEM) age of ewes was 5.0 ± 0.3 years and the interval from the last lambing (post-partum period) was 149.8 ± 0.5 days. The mean body condition score of the ewes (from 1 to 5 where 1 = emaciated and 5 = obese) was 2.7 ± 0.0 (Morada Nova = 2.8 ± 0.1, Santa Inês = 2.8 ± 0.1, and Somalis Brasileira = 2.5 ± 0.1), and the mean body weight at the onset of the study was 36.7 ± 1.6 kg (Morada Nova = 33.9 ± 1.7 kg, Santa Inês = 46.7 ± 1.3 kg, and Somalis Brasileira = 28.6 ± 0.9 kg). The animals were kept in pens and received silage grass and balanced feed in addition to mineral salt licks and water provided ad libitum.

2.2. Superovulatory protocol and mating

The estrus synchronization protocol used in this study was based on that described by (Fonseca et al., 2021). In brief, on a random day of the estrous cycle (Day 0), all ewes received an intravaginal device impregnated with 0.33 g of progesterone (EAZI-BREED CIDR®, Zoetis, São Paulo, SP, Brazil), which was left in place for nine days. The superovulatory treatment with 133 mg of porcine follicle-stimulating hormone (pFSH; Folltropin®-V, Bioniche Animal Health Canada Inc., Belleville, ON, Canada) began 60 h before CIDR® removal (Day 7) and consisted of six consecutive injections of decreasing pFSH doses (25%, 25%, 15%, 15%, 10% and 10% of a total dose) given every 12 h. D-cloprostenol (37.5 μg; Prolise®; Tecnopec, São Paulo, SP, Brazil) was administered i.m. concurrently with the 5th and 6th dose of pFSH. On Day 10 (12 h after CIDR® removal), estrus detection with vasectomized rams commenced; the ewes and a teaser ram were then kept together twice daily (8 a.m. and 4 p.m.) for 30 min, for three days. All females showing signs of behavioral estrus were mated naturally with rams of the homologous breed that had previously been subjected to the breeding soundness (andrological) evaluation; each ewe was mated three times at 12-h intervals, with the first mating immediately after the confirmation of estrus. To prevent premature regression of the corpora lutea (CL), three equal doses (6.75 mg) of flunixin meglumine (Banamine®, MSD Saúde Animal, Cruzeiro, SP, Brazil) were administered i.m. on Days 12 (72 h after CIDR® removal), 13 and 15 of the superovulatory protocol, respectively (Fig. 1).

2.3. Ultrasound assessment

Transrectal ovarian ultrasonography was performed on Day 11 (36 h after CIDR® removal; Fig. 2A) and Day 15 (12 h before embryo collection; early luteal phase) in all animals studied (Fig. 2B). Ultrasonographic examinations were done with the Mindray Z5 Vet ultrasound scanner (Shenzhen, Guandong, China) equipped with a multi-frequency transrectal transducer (at 8.5-MHz frequency). All visible antral follicles $\geq$ 2 mm in diameter and detectable corpora lutea (CL) were measured and the two dimensions (vertical and horizontal) were used to calculate their mean diameters. Color Doppler ultrasonography was performed to detect and record ovarian blood perfusion (i.e., antral follicular blood supply on Day 11 and total follicular/luteal vascularization on Day 15). The standardized settings of the ultrasound equipment were as follows: B-mode (depth: 4.6 cm; main gain: 90%; frame rate: 55 frames/sec, and dynamic range: 120 dB) and color Doppler mode (frequency: 5.7 MHz, main gain: 50%, color wall filter: 183 Hz, and pulse repetition frequency: 1.1 kHz).

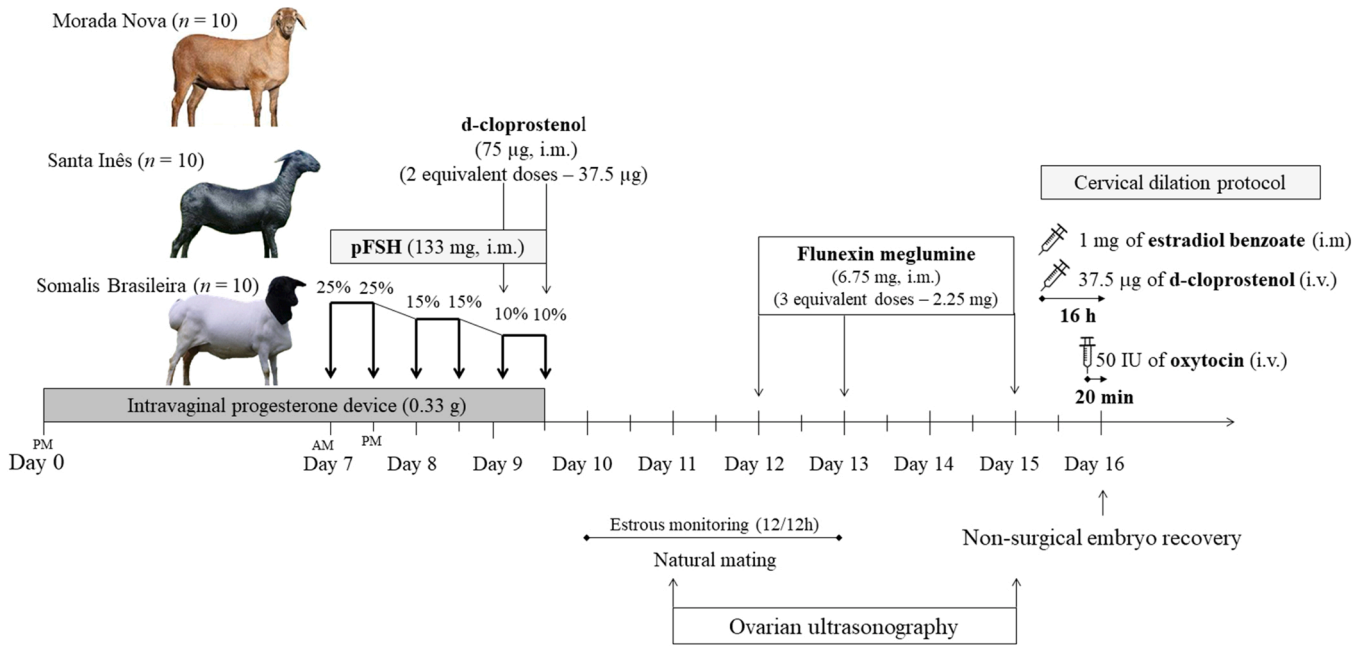

Fig. 1. Schematic representation of the experimental procedures employed to evaluate the effect of the breed (Morada Nova, Santa Inês and Somalis Brasileira) on superovulatory outcomes.

2.4. Non-surgical embryo recovery

Non-surgical embryo recovery (NSER) was conducted seven days after CIDR® removal. The NSER technique used in this study has previously been described by Fonseca et al. (2019) and Figueira et al. (2020). Briefly, all ewes were first subjected to a hormonal cervical dilation protocol consisting of 37.5 µg of D-cloprostenol (Prolise®; Tecnopec, São Paulo, SP, Brazil) and 1 mg of estradiol benzoate (EB; Estrogin®, BIOFARM, São Paulo, Brazil) i.m. given 16 h prior to embryo collection, and 50 IU of oxytocin i.v. (OT; Oxytocin forte®, UCB, São Paulo, SP, Brazil) given 20 min before NSER procedure. Recovered liquid was filtered and examined under stereomicroscope, and all retrieved structures were classified according to guidelines summarized in the International Embryo Society Transfer manual (Stringfellow and Givens, 2010).

2.5. Analyses of ultrasound images

The blood perfusion of the ovarian structures and parenchyma was semi-quantitatively evaluated. The entire cross-sectional area of the ovaries was visible within the power Doppler sample box. We prepared video sequences of four- and five-seconds maximum duration. Ultrasonic videos were exported in an AVI format to a computer and on the digitized ultrasonographic images, the region of interest was defined by the examiner, the frame containing ovarian cross section with the strongest Doppler signal plus one frame directly preceding it and one directly following it, were stored in a JPG format for image analysis. The colored area in relation to the total area of the tissue was measured using the computer Image Pro Plus 3.0.1 software (Media Cybernetics, Silver Spring, MA, USA). For each ultrasonographic variable, the average of the three frames was calculated. The variables analyzed were as follows: total ovarian area (TA), color Doppler area (DA), and high-velocity blood flow area (HVBFA, 0.055–0.11 m/s). The latter was determined as previously described by Oliveira et al. (2017). Briefly, the scale bar depicting a full range of recordable blood flow velocities was divided into 64 lines of visibly distinctive colors, and the top one-quarter (16 rows) and the bottom one-quarter (16 rows) of the scale bar were selected to trace high-velocity color pixels ($\geq$ 0.11 m/s).

2.6. End points and statistical analyses

The following variables were recorded or calculated for all ewes studied on Day 11: (ovarian data) total number of all antral follicles $\geq$ 2 mm in diameter; diameter of the largest follicle (mm); number of large antral follicles ($\geq$ 6 mm in diameter); average diameter of large follicles (mm); number of medium-sized follicles (4.0–5.99 mm diameter); average diameter of medium follicles (mm); number of small follicles ($\leq$ 3.99 mm); average diameter of small follicles (mm); ovarian cross-sectional area (mm^2); blood flow or color Doppler area (mm^2); high-velocity blood flow area (HVBFA) (mm^2); mean percentage of color Doppler area (DA/TA $\times$ 100%); percentage of HVBFA in relation to the total ovarian area (HVBFA/TA $\times$ 100%); percentage of HVBFA in relation to the total Doppler area (HVBFA/DA $\times$ 100%); (behavioral data): estrus response (percentage of ewes in estrus after CIDR® removal); interval from CIDR® removal to the onset of estrus (h); and duration of behavioral estrus (h). The following variables were recorded for all ewes studied on Day 15 (ovarian data) and 12 h before non-surgical embryo recovery (superovulatory responses): (ovarian data) number of CL; number of luteinized unovulated follicles (LUFs; defined as unruptured, round-shaped and cavitated antral follicles measuring 5–10 mm in diameter; superovulatory (SOV) status (score 0: 0–3 CL; ewes that had poor responses and/or did not respond to superovulatory treatment; score 1: 4–10 CL; score 2: 11–15 CL; and score 3: $\geq$ 16 CL); ovarian cross-sectional area (mm^2); blood flow or color Doppler area (mm^2); HVBFA area (mm^2); average percentage of Doppler area (DA/TA $\times$ 100%); percentage of HVBFA in relation to the total ovarian area (HVBFA/TA $\times$ 100%); and percentage of HVBFA in relation to total Doppler area (HVBFA/DA $\times$ 100%); (superovulatory responses): number of recovered structures; recovery rate (number of all recovered structures/number of CL $\times$ 100%); number of viable embryos (number of embryos classified from I to III according to the guidelines summarized in the International Embryo Society Transfer manual); viability rate (number of viable embryos/number of recovered structures $\times$ 100%); and embryo recovery score (score 0: no collection; score 1: 0 viable embryos; score 2: 1–7 viable embryos; score 3: 8–14 viable embryos; and score 4: $\geq$ 15 viable embryos).

Data normality was checked with the Shapiro-Wilk test and the homogeneity of variance was tested with the Bartlett's test. The variables

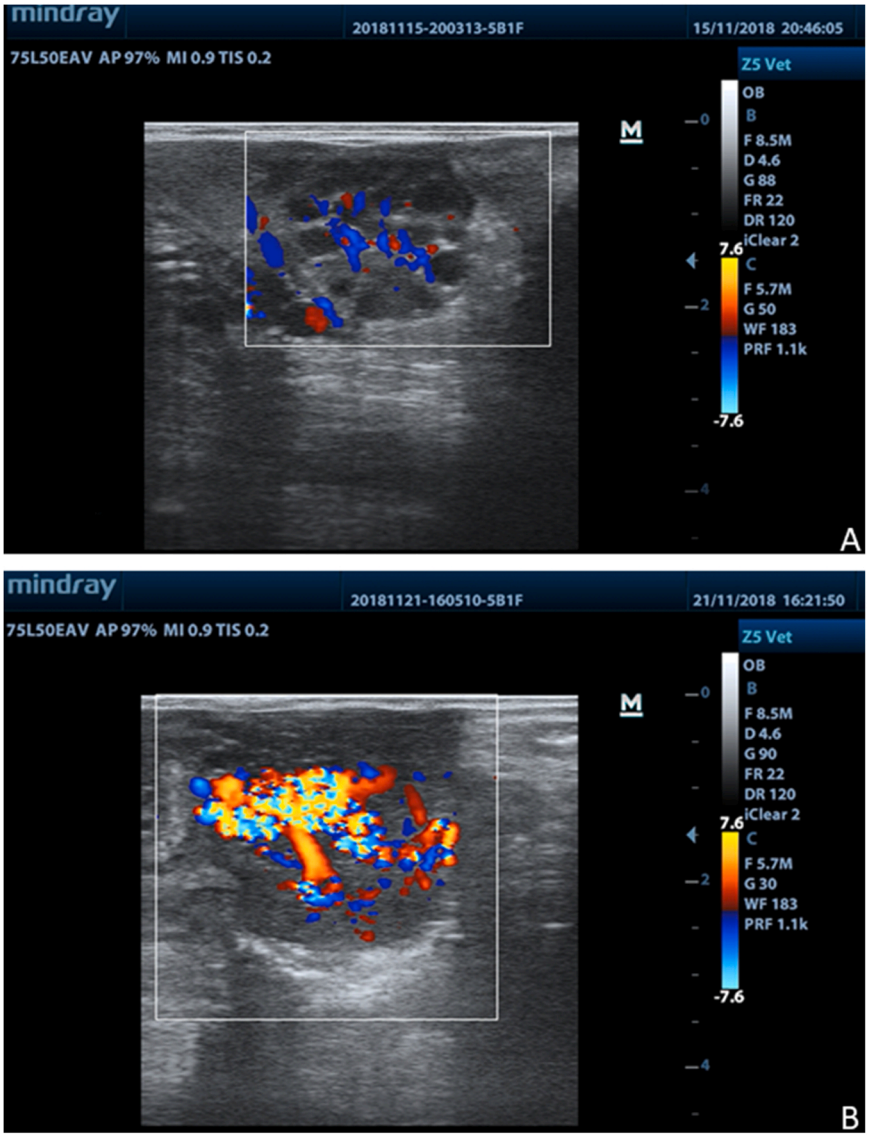

Fig. 2. Color Doppler ultrasonograms of ovarian blood perfusion characteristics in three native Brazilian breeds of sheep (Morada Nova, Santa Inês and Somalis Brasileira) subjected to a 9-day progesterone-based estrus synchronization protocol coupled with 133 mg of pFSH administrated in six decreasing doses (starting 60 h before intravaginal device removal). A. Ovarian ultrasonogram captured on Day 11 in a Santa Inês donor ewe; B. An ultrassonografic image recorded on Day 15 in a Santa Inês ewe.

obtained at sequential time points were analyzed by PROC MIXED of SAS (SAS Inst., Inc., Cary, NC, USA). In the statistical model used, the fixed effects of breed, days of ultrasound evaluation and their interactions were included for comparison of parametric data using the analysis of variance of repeated bidirectional measurements (ANOVA) and the Tukey (post-ANOVA) test. All data sets that did not pass the normal distribution/equal variance test were analyzed using the Whitney-Mann (non-parametric) test. All single time-point variables were compared among groups (breed effect) by one-way ANOVA and Tukey test. The Pearson Product Moment correlation was performed among antral follicular data/quantitative color Doppler measurements on Days 11 and superovulatory responses with GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA). All results were presented as mean ± standard error of the mean (SEM). The value of $P \leq 0.05$ was considered statistically significant.

3. Results

All ewes were in estrus after CIDR® removal. The average interval from CIDR® removal to the onset of estrus was longer ($P < 0.05$) in Somalis Brasileira (33.6 ± 3.5 h) compared with that in Morada Nova (25.0 ± 2.7 h) and Santa Inês (20.8 ± 1.8 h) ewes. The mean duration of behavioral estrus (22.4 ± 1.2 h) did not differ ($P > 0.05$) among the three breeds of ewes studied. On Day 11, the number of medium-sized follicles (4.0–5.99 mm in diameter) was greater ($P < 0.05$) in Santa Inês and Somalis Brasileira compared with that in Morada Nova ewes (Table 1). There were no differences ($P > 0.05$) among the three breeds of sheep in mean follicle numbers or dimensions for small or large ovarian antral follicles detected ultrasonographically on Day 11.

The SOV status of ewes and mean number of CL per ewe were greatest ($P < 0.05$) in Santa Inês ewes and they were greater ($P < 0.05$) in Somalis Brasileira than in Morada Nova ewes, whereas the mean number of CL in superovulated animals (with ≥ 4 CL post-treatment) was greater ($P < 0.05$) in Santa Inês compared with that in Somalis Brasileira and Morada Nova sheep (Table 2). The number of luteinized unovulated follicles, number of all recovered structures, recovery rate, number of viable embryos, viability rate and embryo status did not differ among the three breeds of ewes studied ($P > 0.05$; Table 2).

There was a significant interaction between Breed and Day of ultrasound examination for the ovarian cross-sectional area ($P < 0.05$), blood flow area ($P = 0.05$), and the percentage of color Doppler area ($P < 0.05$; Fig. 2). The ovarian cross-sectional area and blood flow area on Day 15 were greater in Santa Inês and Somalis Brasileira ewes compared with that in Morada Nova ewes ($P < 0.05$), and they were

Table 1
Ovarian antral follicle populations (mean ± SEM) on Day 11 (36 h after CIDR® removal) recorded ultrasonographically in Morada Nova, Santa Inês or Somalis Brasileira ewes that underwent the 9-day progesterone-based estrus synchronization protocol and the 3-day pFSH superovulatory treatment (133 mg of pFSH administrated in six decreasing doses from 60 h before CIDR® removal).

End points	Morada Nova	Santa Inês	Somalis Brasileira	*P* value
Total number of antral follicles ≥ 2 mm	18.7 ± 2.2	31.3 ± 5.5	26.6 ± 3.0	0.08
Diameter of the largest follicle (mm)	5.9 ± 0.5	6.1 ± 0.4	5.4 ± 0.2	0.39
Number of large follicles (≥ 6 mm)	0.9 ± 0.6	1.1 ± 0.5	0.1 ± 0.1	0.25
Average diameter of large follicles (mm)	6.9 ± 0.6	6.9 ± 0.1	6.2 ± 0.0	0.74
Number of medium-sized follicles (4.0–5.99 mm)	3.9 ± 0.8[b]	10.0 ± 1.3[a]	8.8 ± 1.4[a]	0.002
Average diameter of medium-sized follicles (mm)	4.6 ± 0.1	4.6 ± 0.0	4.6 ± 0.1	0.98
Number of small follicles (2.0–3.99 mm)	13.9 ± 1.9	20.2 ± 5.2	17.7 ± 2.1	0.43
Average diameter of small follicles (mm)	3.0 ± 0.1	3.0 ± 0.1	3.0 ± 0.1	0.93

[ab] Within rows, means denoted by different letter superscripts vary significantly.

Table 2
Superovulatory responses and in vivo embryo production (mean ± SEM) in three native Brazilian breeds of ewes that underwent the 9-day progesterone-based estrus synchronization protocol and the 3-day pFSH superovulatory treatment (133 mg of pFSH administrated in six decreasing doses from 60 h before CIDR® removal).

End points	Morada Nova	Santa Inês	Somalis Brasileira	*P* value
SOV status*	0.8 ± 0.3[c]	2.4 ± 0.2[a]	1.5 ± 0.2[b]	< 0.0001
Number of CL in all ewes	5.5 ± 1.6[c] (1–18)	15.3 ± 1.4[a] (8–24)	10.4 ± 0.8[b] (6–14)	< 0.0001
Number of CL in ewes with ≥ 4 CL	7.8 ± 2.2[b] (4–18)	15.3 ± 1.4[a] (4–24)	10.4 ± 0.8[b] (4–14)	0.004
Number of luteinized unovulated follicles	1.8 ± 0.6 (0–4)	0.9 ± 0.3 (0–3)	1.3 ± 0.6 (0–6)	0.45
Number of all recovered structures	7.6 ± 2.2	13.3 ± 5.3	6.4 ± 1.1	0.37
Recovery rate (%)	79.0 ± 20.3	73.8 ± 22.9	62.0 ± 10.6	0.81
Number of viable embryos	6.6 ± 2.7	10.8 ± 5.1	5.3 ± 1.4	0.53
Viability rate (%)	66.7 ± 21.1	70.7 ± 12.1	76.0 ± 13.3	0.91
Embryo recovery score* *	1.1 ± 0.4	2.1 ± 0.4	2.1 ± 0.3	0.11

* Score 0: 0–3 CL; score 1: 4–10 CL; score 2: 11–15 CL; and score 3: ≥ 16 CL.
* * Score 0: embryos not collected; score 1: 0 viable embryos; score 2: 1–7 viable embryos; score 3: 8–14 viable embryos; and score 4: ≥ 15 viable embryos.
[ab] Within rows, means denoted by different letter superscripts vary significantly.

greater on Day 15 than on Day 11 in all breeds studied ($P < 0.05$). The percentage of Doppler area was greater ($P < 0.05$) in Santa Inês compared with that in Morada Nova ewes, and on Day 15 this variable in Morada Nova ewes was less ($P < 0.05$) compared with those in the other two breeds. The number of high-velocity blood flow pixels, percentage of HVBFA in relation to the total ovarian area, and percentage of HVBFA in relation to total Doppler area were all greater ($P < 0.05$) on Day 15 compared with that in Day 11 in the three genotypes of ewes studied. Fig. 3.

Significant correlations among ovarian data recorded on Day 11 (antral follicle counts and color Doppler indices) and superovulatory responses in the ewes of the present study are summarized in Table 3. The number of ultrasonographically detected CL (Day 15) was positively correlated with the number of small antral follicles on Day 11 in all three breeds of ewes studied. In addition, in Morada Nova and Santa Inês ewes, the ovulatory response was also directly related to the total number of all antral follicles ≥ 2 mm in diameter. The number of all structures recovered by NSER was positively correlated with the number of medium-sized antral follicles (4.0–5.99 mm) as well as with ovarian color Doppler area (DA) and average percentage of Doppler area (DA/TA × 100%) in Morada Nova ewes, but only with the total number of antral follicles and small follicle counts in Santa Inês ewes. The number of viable embryos was positively correlated with DA in Morada Nova ewes but in Santa Inês sheep it was directly related to the total number of antral follicles, the number of small follicles, DA and DA/TA × 100%. Significant correlations among ovarian blood perfusion variables on Day 11 and superovulatory responses were observed only in Morada Nova and Santa Inês ewes.

4. Discussion

Breeds of sheep varying in prolificacy can respond differently to identical SOV protocols (Figueira et al., 2020; Dufour et al., 2000). However, to the best of the authors' knowledge, this is the first comparative study evaluating the superovulatory response in three naturalized Brazilian breeds of sheep raised in the similar climatic conditions and subjected to the same superovulatory and embryo collection protocols. There was a difference among the three breeds studied in the ultrasonographically determined number of medium-sized follicles (4.0–5.99 mm) on Day 11 and of CL (ovulation rate) on Day 15 (i.e., 36 h after CIDR® removal or 12 h before embryo recovery, respectively), but the embryo yields after NSER did not vary between the three genotypes of ewes.

In the present study, the Santa Inês had a greater ovulatory response compared with the two other breeds, and the Morada Nova showed the lowest response of all three genotypes. In previous studies with a 14-day progesterone protocol, superovulated Morada Nova ewes had greater numbers of CL, all recovered structures and viable embryos compared with Somalis Brasileira (Brasil et al., 2018). Rebolledo et al. (2017) reported that non-prolific Dorper ewes had better superovulatory responses compared to the breeds with higher ovulation rates such as Blackbelly, Katahdin or Pelibuey. Based on the data collected over the 4 years, between October and December each year, in Sobral, CE, Brazil, Morada Nova ewes had on the average a greater ovulation rate (1.82) than Somalis Brasileira (1.39) and Santa Inês ewes (1.31; Rajab et al., 1992). It is, therefore, unlikely that the present SOV responses were due mainly to the ewes' inherent prolificacy. Such a variability could be caused, at least in part, by the differences in body weight among the three breeds of ewes studied (Santa Inês > Morada Nova > Somalis Brasileira). It has been shown that lower total doses of exogenous FSH may elicit better and less variable superovulatory responses in ewes (Maciel et al., 2017). By that logic, higher ovulatory responses may have been associated with the larger body weight of Santa Inês ewes and fewer ovulations were observed in significantly lighter Morada Nova and Somalis Brasileira donors receiving the same dose of pFSH. However, in the body weight was a primary determinant of ovulatory responses to the same total dose of exogenous FSH, the Morada Nova would have had a higher ovulation rate compared with that of Somalis Brasileira donors, whereas and the present results were the opposite.

The expression of prolificacy genes encoding bone morphogenetic protein 15 (BMP15) and growth and differentiation factor 9 (GDF9) dictates ovulation rates in sheep (Moore et al., 2004). Bone morphogenetic protein 15 and GDF9 are oocyte-synthesized proteins with profound effects on fertility in mammals (Wilson et al., 2001). Both these factors regulate follicular cell proliferation and differentiation. Spontaneous mutations inactivate the BMP15 and GDF9 genes in heterozygous animals, promoting a reduction in protein levels and enhanced ovulatory responses; alternatively, homozygous animals are sterile (Galloway

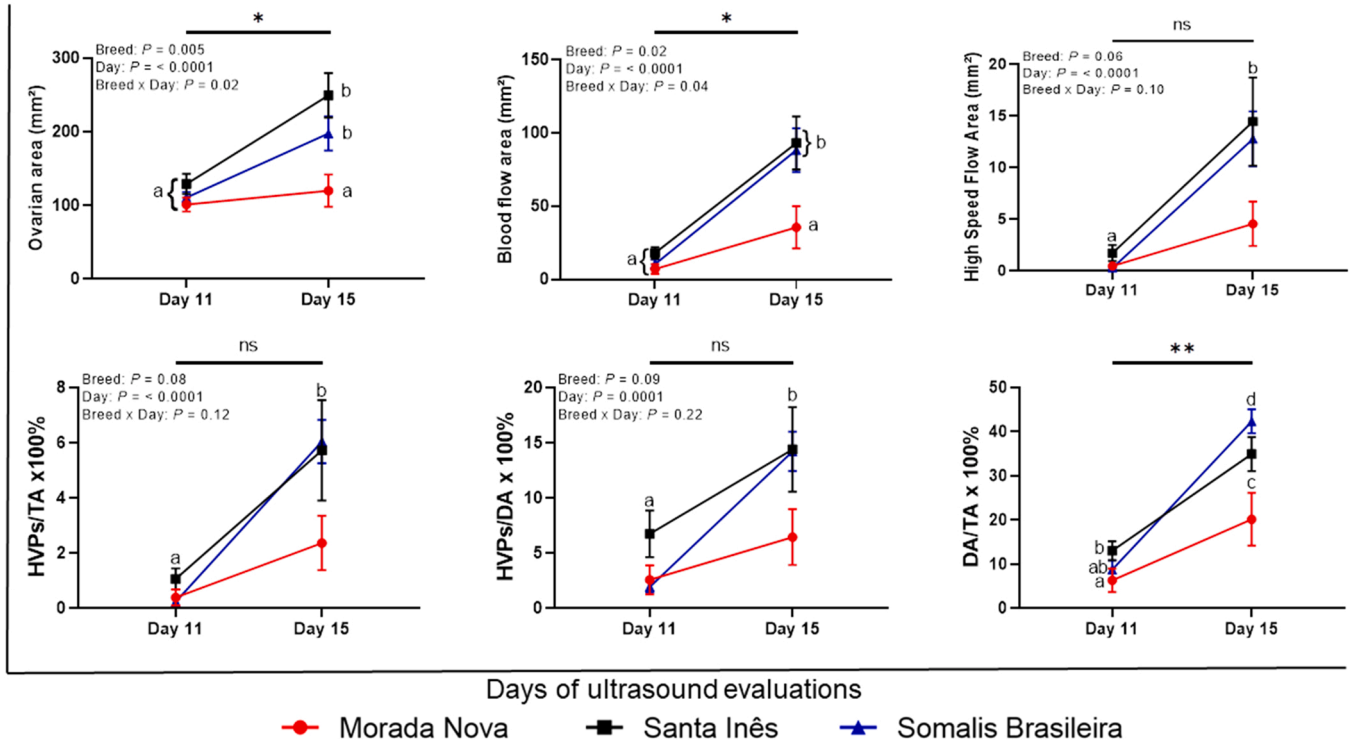

Fig. 3. Ovarian blood perfusion characteristics (mean ± SEM) recorded on Days 11 ((36 h after CIDR® removal) and 15 (12 h before embryo recovery) in native Brazilian sheep (Morada Nova - red, Santa Inês - black and Somalis Brasileira - blue) subjected to a 9-day progesterone-based estrus synchronization protocol coupled with 133 mg of pFSH administrated in six decreasing doses (starting 60 h before intravaginal device removal). TA: total ovarian area, DA: color Doppler area, and HVBFA: high-velocity blood flow area. P values for Breed vs. Day intercation were as follows: ns - 0.12, * - 0.033, ** - 0.002 and *** - < 0.001. Different letters (a-d) denote stastically significant differences between the breeds or over time. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
A summary of significant correlations among ovarian data determined ultrasonographically on Day 11 (36 h after CIDR® removal) and quantitative superovulatory responses in three indigenous Brazilian breeds of sheep subjected to the same estrus synchronization and hormonal ovarian superstimulation protocols. r: coefficient of correlation.

Input variable	Output variable	r	*P* value
Morada Nova			
Total number of antral follicles ≥ 2 mm	Number of CL	0.83	0.003
Number of small follicles (2.0–3.99 mm)		0.66	0.04
Color Doppler area (DA) (mm^2)		0.81	0.005
DA/Total cross-sectional area (TA) × 100%		0.85	0.002
Number of medium-sized follicles (4.0–5.99 mm)	Number of all recovered structures	0.98	0.004
DA		0.97	0.007
DA/TA × 100%		0.96	0.008
DA	Number of viable embryos	0.98	0.003
Santa Inês			
Total number of antral follicles	Number of CL	0.73	0.02
Number of small follicles		0.71	0.02
Total number of antral follicles	Number of all recovered structures	0.75	0.02
Number of small follicles		0.78	0.01
Total number of antral follicles	Number of viable embryos	0.79	0.01
Number of small follicles		0.83	0.006
DA		0.67	0.05
DA/TA × 100%		0.66	0.05
Somalis Brasileira			
Number of small follicles	Number of CL	0.75	0.01

et al., 2000; Hanrahan et al., 2004). The genetic alterations involving BMP15 and GDF9 are closely related to the occurrence of the polymorphism in FecXI and FecXH (Galloway et al., 2000), FecBB (Mulsant et al., 2001; Souza et al., 2001), FecGH (Hanrahan et al., 2004), and FecGE (Silva et al., 2011). The FecGE detected in Santa Inês ewe is a gene variant that represents a new reproductive phenotype; the homozygotes are not sterile and have increased prolificacy, which differs from other documented genetic variants of GDF9 (Silva et al., 2011). Conversely, Morada Nova and Santa Inês in Northeastern Brazil had the average of 2.44 and 2.13 offspring/birth, respectively, although they lack any of the prolificacy gene mutations (Holanda et al., 2017). More studies are needed to elucidate the influence of prolificacy genes on ovarian activity and superovulatory yields in ewes.

B-mode and color Doppler sonography is a practical tool that can be used in a commercial and research setting to aid in predicting ovarian responses and superovulatory yields in sheep (Oliveira et. al, 2014). Apart from the Morada Nova breed in this study, the ovarian area and total blood flow were greater in ovaries containing CL compared with those containing potential ovulatory follicles. This agrees with earlier studies by Acosta et al. (2005), Matsui and Miyamoto (2009), and Lansbergen (2013) who demonstrated that an increase in luteal tissue volume occurs concurrently with the formation of elaborate vascular network.

The present results of correlation analyses indicate that ultrasonographic enumeration of ovarian antral follicles following the removal of vaginal progesterone-releasing devices can be an early predictor of the superovulatory response in ewes. Quite unexpectedly, however, mainly small follicle numbers recorded 36 h after CIDR® withdrawal were indicative of the ensuing ovulation rates in the ewes of the present study. Moreover, both the total follicle counts, and small follicle numbers were correlated with the numbers of CL in Morada Nova and Santa Inês ewes,

but in Somalis Brasileira only small follicle numbers were positively correlated with ovulatory responses. Previously, Ammoun et al. (2006) have reported that the number of 4-mm follicles at the time of medroxyprogesterone acetate sponge withdrawal is indicative of superovulatory responses in ewes. Therefore, the present study identified a new ovarian marker for determining which animals should be subjected to embryo recovery. Another intriguing observation was that antral follicle numbers were a good predictor of NSER efficacy (total numbers of recovered structures) and of viable embryos recovered in superovulated Morada Nova and Santa Inês ewes, but not in Somalis Brasileira donors.

Ovarian blood supply is closely related to the follicular growth and physiological status (Matsui and Miyamoto, 2009). Strong correlations between the ovarian blood perfusion indices and superovulatory responses/embryo production were noted; however, such correlations were not consistent among the three genotypes of sheep studied. In fact, the positive correlations between the ovarian blood perfusion on Day 11 and superovulatory outcomes were restricted to Morada Nova and Santa Inês ewes. Oliveira et al. (2014) showed that antral follicular blood flow (color Doppler) on the last day of the 4-day superovulatory pFSH protocol was directly related to the number and percentage of unfertilized eggs in Santa Inês ewes. In addition, high velocity follicular blood flow (0.055–0.11 m/s) on different days of the protocol was associated with the embryo recovery and viability rate, and percentages of degenerated embryos in superovulated Santa Inês ewes (Oliveira et al., 2017). However, elevated blood flow to the preovulatory follicles was associated with increased oocyte recovery in superovulated cows (Siddiqui et al., 2009). It is feasible that both ovarian and follicular blood perfusion indices recorded with the use of color Doppler sonography at different times during and after the application of the SOV protocol can be used to predict the superovulatory response in ewes. Moreover, such associations may be species- and/or breed-specific, which warrants further studies of the causative relationships between antral follicular health status/vascularity, ovulatory ability, and oocyte competence.

5. Conclusion

There were inter-breed differences in the number of medium-sized antral follicles on Day 11 (36 h following estrus synchronization with CIDR®) and the number of CLs on Day 15 (one day before embryo flushing) in three Brazilian native breeds of sheep (Morada Nova, Santa Inês and Somalis Brasileira); however, the number of viable embryos recovered using NSER did not vary among the three breeds studied. Ovarian blood perfusion increased from just before ovulation to CL formation in superovulated ewes. The number of small antral follicles on Day 11 is a consistent and reliable marker of ovulatory responses while the ovarian parenchymal Color Doppler signal on Day 11 is a predictor of viable embryo yields in hormonally superstimulated Morada Nova and Santa Inês ewes. Environmental and intrinsic factors underlying the differences in antral follicular development, and associations between ovarian blood flow indices and ensuing superovulatory outcomes in different breeds of sheep warrant further studies.

Acknowledgements

This work was supported by the Embrapa Caprinos e Ovinos, Brazil [02.13.06.026.00.02; 02.13.06.026.00.04; Minas Gerais Research Foundation - FAPEMIG, Brazil [00201-17; Coordination for the Improvement of Higher Education - CAPES, Brazil; and the United National Council for Scientific and Technological Development – CNPq, Brazil.

Authors' contributions

MEFO and JFF designed the present study. GBV, MSDL, RITPB, JFF, KMS, AWUM, AFR and MAFP helped with animal management and data collection. MAFP, MEFO and PMB analyzed the data. MAFP and MEFO wrote the first version of the manuscript. MAFP and PMB wrote the last version of the manuscript. PMB, MEFO, JMGSF and JFF revised and approved the final version of the manuscript.

Conflict of interest

None of the authors has a conflict of interest to declare.

References

Acosta, T.J., Hayashi, K.G., Matsui, M., Miyamoto, A., 2005. Changes in follicular vascularity during the first follicular wave in lactating cows. J. Reprod. Dev. 51, 273–280. https://doi.org/10.1262/jrd.16092.

Ammoun, I., Encinas, T., Veiga-Lopez, A., Ros, J.M., Contreras, I., Gonzalez-Añover, P., Cocero, M.J., McNeilly, A.S., Gonzalez-Bulnes, A., 2006. Effects of breed on kinetics of ovine FSH and ovarian response in superovulated sheep. Theriogenology 66, 896–905. https://doi.org/10.1016/j.theriogenology.2006.02.024.

Baldassarre, H., Karatzas, C.N., 2004. Advanced assisted reproduction technologies (ART) in goats. Anim. Reprod. Sci. 82–83, 255–266. https://doi.org/10.1016/j.anireprosci.2004.04.027.

Bari, F., Khalid, M., Wolf, B., Haresign, W., Murray, A., Merrell, B., 2001. The repeatability of superovulatory response and embryo recovery in sheep. Theriogenology 56, 147–155. https://doi.org/10.1016/S0093-691X(01)00550-7.

Bartlewski, P.M., 2019. Recent advances in superovulation in sheep. Revista Brasileira de Reprodução. Animal 43, 126–128.

Bartlewski, P.M., Seaton, P., Franco Oliveira, M.E., Kridli, R.T., Murawski, M., Schwarz, T., 2016. Intrinsic determinants and predictors of superovulatory yields in sheep: Circulating concentrations of reproductive hormones, ovarian status, and antral follicular blood flow. Theriogenology 86, 130–143. https://doi.org/10.1016/j.theriogenology.2016.04.024.

Brasil, O.O., Moreira, N.H., Silva, T.A.S.N., Silva, B.D.M., Nascimento, N.V., Facó, O., Ramos, A.F., 2018. Produção de embriões em ovinos Morada Nova e Somalis Brasileira. Arq. Bras. De. Med. Veter e Zootec. 68, 1390–1394 https://doi.org/http://de.doi.ori/10.1590/1678-4162-8428.

Célia, Lessa, Ramos, T.B., Rici, P., Bombonato, R.E.G., Ambrósio, C.E, P.P., 2012. Effect of flunixin meglumine and hcg at commercial programs for multiple ovulation and embryo transfer (moet) in sheeP. Arch. Vet. Sci. 17, 63–69.

Clark, A.R., Stokes, Y.M., 2011. Follicle structure influences the availability of oxygen to the oocyte in antral follicles. Comput. Math. Methods Med. 2011. https://doi.org/10.1155/2011/287186.

Dufour, J.J., Cognié, Y., Mermillod, P., Mariana, J.C., Romain, R.F., 2000. Effects of the Booroola Fec gene on ovarian follicular populations in superovulated Romanov ewes pretreated with a GnRH antagonist. J. Reprod. Fertil. 118, 85–94. https://doi.org/10.1530/reprod/118.1.85.

Figueira, L.M., Alves, N.G., Souza-Fabjan, J.M.G., Oliveira, M.E.F., Lima, R.R., Souza, G. N., Fonseca, J.F., 2020. Preovulatory follicular dynamics, ovulatory response and embryo yield in Lacaune ewes subjected to synchronous estrus induction protocols and non-surgical embryo recovery. Theriogenology 145, 238–246. https://doi.org/10.1016/j.theriogenology.2019.11.004.

Fonseca, J.F., Zambrini, F.N., Guimarães, J.D., Silva, M.R., Oliveira, M.E.F., Brandão, F. Z., Bartlewski, P.M., Souza-Fabjan, J.M.G., 2019. Combined treatment with oestradiol benzoate, d-cloprostenol and oxytocin permits cervical dilation and nonsurgical embryo recovery in ewes. Reprod. Domest. Anim. 54, 118–125. https://doi.org/10.1111/rda.13318.

Fonseca, J.F., Vergani, G.B., Lima, M.S.D., Silva, K.M., Monteiro, A.W.U., Ramos, A.F., Alves, B.R.C., Souza-Fabjan, J.M.G., Oliveira, M.E.F., Batista, R.I.T.P., 2021. Nonsurgical embryo recovery as a feasible tool for supporting embryo biobanks of locally adapted brazilian sheep and goats. Biopreserv Biobank. https://doi.org/10.1089/bio.2021.0066.

Galloway, S.M., McNatty, K.P., Cambridge, L.M., Laitinen, M.P.E., Juengel, J.L., Jokiranta, T.S., McLaren, R.J., Luiro, K., Dodds, K.G., Montgomery, G.W., Beattie, A. E., Davis, G.H., Ritvos, O., 2000. Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. Nat. Genet. 25, 279–283. https://doi.org/10.1038/77033.

Hanrahan, J.P., Gregan, S.M., Mulsant, P., Mullen, M., Davis, G.H., Powell, R., Galloway, S.M., 2004. Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and belclare sheep (Ovis aries). Biol. Reprod. 70, 900–909. https://doi.org/10.1095/biolreprod.103.023093.

Holanda, G.M.L., Oliveira, J.C., Silva, D.M.F., Rocha, S.S.N., Pandolfi, V., Adrião, M., Wischral, A., 2017. Survey of mutations in prolificacy genes in Santa Ines and Morada Nova sheep. Arq. Bras. De. Med. Vet. e Zootec. 69, 1047–1053. https://doi.org/10.1590/1678-4162-9339.

Lansbergen, M., 2013. Bovine follicular and luteal blood flow during the estrous cycle Clinical evalutation of the ultrasound Doppler technology, pp. 1–39.

Lass, A., Brinsden, P., 1999. The role of ovarian volume in reproductive medicine. Hum. Reprod. Update 5, 256–266. https://doi.org/10.1093/humupd/5.3.256.

Maciel, G.S., Rodriguez, M.G.K., Da Silva, P.D.A., Nociti, R.P., Uscategui, R.A.R., Santos, V.J.C., Feliciano, M.A.R., Vicente, W.R.R., Oliveira, M.E.F., 2017. Ovarian superstimulation treatment for multiple ovulation and embryo transfer programs in sheep. Investigação 16, 30–36. https://doi.org/10.26843/investigacao.v16i8.1888.

Matsui, M., Miyamoto, A., 2009. Evaluation of ovarian blood flow by colour Doppler ultrasound: practical use for reproductive management in the cow. Vet. J. 181, 232–240. https://doi.org/10.1016/j.tvjl.2008.02.027.

Moore, R.K., Erickson, G.F., Shimasaki, S., 2004. Are BMP-15 and GDF-9 primary determinants of ovulation quota in mammals? Trends Endocrinol. Metab. 15, 356–361. https://doi.org/10.1016/j.tem.2004.08.008.

Mulsant, P., Lecerf, F., Fabre, S., Schibler, L., Monget, P., Lanneluc, I., Pisselet, C., Riquet, J., Monniaux, D., Callebaut, I., Cribiu, E., Thimonier, J., Teyssier, J., Bodin, L., Cognié, Y., Chitour, N., Elsen, J.M., 2001. Mutation in bone morphogenetic protein receptor-IB is associated with increased ovulation rate in Booroola Mérino ewes. Proc. Natl. Acad. Sci. USA 98, 5104–5109. https://doi.org/10.1073/pnas.091577598.

Oliveira, M.E.F., Feliciano, M.A.R., D'Amato, C.C., Oliveira, L.G., Bicudo, S.D., Fonseca, J.F., Vicente, W.R.R., Visco, E., Bartlewski, P.M., 2014. Correlations between ovarian follicular blood flow and superovulatory responses in ewes. Anim. Reprod. Sci. 144, 30–37. https://doi.org/10.1016/j.anireprosci.2013.10.012.

Oliveira, M.E.F., Bartlewski, P.M., Jankowski, N., Padilha-Nakaghi, L.C., Oliveira, L.G., Bicudo, S.D., Fonseca, J.F., Vicente, W.R.R., 2017. Relationship of antral follicular blood flow velocity to superovulatory responses in ewes. Anim. Reprod. Sci. 182, 48–55. https://doi.org/10.1016/j.anireprosci.2017.04.009.

Pinto, P.H.N., Balaro, M.F.A., Saraiva, H.F.R.D.A., Brair, V.L., Alfradique, V.A.P., Côrtes, L.R., Cosentino, I.O., Souza-Fabjan, J.M.G., Fonseca, J.F., Da, Brandão, F.Z., 2020. Successive in vivo embryo production in Santa Inês sheep. Anim. Prod. Sci. 60, 497–502. https://doi.org/10.1071/AN18740.

Rajab, M.H., Cartwright, T.C., Dahm, P.F., Figueiredo, E.A., 1992. Performance of three tropical hair sheep breeds. J. Anim. Sci. 70, 3351–3359. https://doi.org/10.2527/1992.70113351x.

Rebolledo, Á.D., Manzanero, G.V., Romero, A.A., Franco, J.Q., Rodriguez, J.B., Lorca, J. R., Ugalde, J.R., 2017. Follicular population at the onset of a superovulatory treatment and ovarian response in hair ewes. Rom. Biotechnol. Lett. 22, 12427–12431.

Siddiqui, M.A.R., Gastal, E.L., Gastal, M.O., Almamun, M., Beg, M.A., Ginther, O.J., 2009. Relationship of vascular perfusion of the wall of the preovulatory follicle to in vitro fertilisation and embryo development in heifers. Reproduction 137, 689–697. https://doi.org/10.1530/REP-08-0403.

Silva, B.D.M., Castro, E.A., Souza, C.J.H., Paiva, S.R., Sartori, R., Franco, M.M., Azevedo, H.C., Silva, T.A.S.N., Vieira, A.M.C., Neves, J.P., Melo, E.O., 2011. A new polymorphism in the growth and differentiation factor 9 (GDF9) gene is associated with increased ovulation rate and prolificacy in homozygous sheep. Anim. Genet. 42, 89–92. https://doi.org/10.1111/j.1365-2052.2010.02078.x.

Souza, C.J.H., MacDougall, C., Campbell, B.K., McNeilly, A.S., Baird, D.T., 2001. The Booroola (FecB) phenotype is associated with a mutation in the bone morphogenetic receptor type 1 B (BMPR1B) gene. J. Endocrinol. 169, 3–8. https://doi.org/10.1677/joe.0.169R001.

Van Blerkom, J., Antczak, M., Schrader, R., 1997. The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: Association with vascular endothelial growth factor levels and perifollicular blood flow characteristics. Hum. Reprod. 12, 1047–1055. https://doi.org/10.1093/humrep/12.5.1047.

Wilson, T., Wu, X.Y., Juengel, J.L., Ross, I.K., Lumsden, J.M., Lord, E.A., Dodds, K.G., Walling, G.A., McEwan, J.C., O'Connell, A.R., McNatty, K.P., Montgomery, G.W., 2001. Highly prolific Booroola sheep have a mutation in the intracellular kinase domain of bone morphogenetic protein IB receptor (ALK-6) that is expressed in both oocytes and granulosa cells. Biol. Reprod. 64, 1225–1235. https://doi.org/10.1095/biolreprod64.4.1225.