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Anti-Müllerian hormone and antral follicle count are more effective for selecting ewes with good potential for in vivo embryo production than the presence of *FecG*^{E} mutation or eCG pre-selection tests

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ABSTRACT

This study aims to compare four different methods for selecting high responding sheep donors for in vivo embryo production. These methods include a pre-selection eCG test (eCG), antral follicle count (AFC), plasma anti-Müllerian hormone measurement (AMH) and genotyping for the presence of the *FecG*^{E} mutation (a polymorphism in the *GDF9* gene associated with increased ovulation rate). Santa Ines ewe lambs (n = 25) underwent superovulation (SOV) with 800 IU equine chorionic gonadotropin (eCG) and the corpus luteum (CL) count was recorded by laparoscopy after eight days. At the $D0_{eCG}$, blood samples for AMH and genotyping analysis were collected. Twenty-one days after the end of the eCG test, the same animals underwent SOV with 200 mg of FSH, administered in six decreasing doses, and then naturally mated. Immediately before the beginning of the FSH protocol ($D0_{FSH}$), and at the moment of the first FSH dose ($D9_{FSH}$), the AFC was assessed. Plasma AMH was again determined at the $D9_{FSH}$. After each screening process, animals were classified as having a high (HR), or low (LR), potential of response (using specific thresholds for each method). Then, the ewes' response to SOV and embryo yield for each screening method, classified as HR or LR, were compared. Animals classified as HR by AFC (HR_{AFC}) and by AMH concentration (HR_{AMH}) at the $D9_{FSH}$, produced more viable embryos than those classified as LR_{AFC} and LR_{AMH} (HR_{AFC} 6.2 ± 3.2 vs LR_{AFC} 2.8 ± 3.0 and HR_{AMH} 6.6 ± 3.6 vs LR_{AMH} 3.0 ± 2.9). Pre-selection tests with eCG and different *FecG*^{E} genotypes, either heterozygous (+/E) or wild type (+/+), were unable to discriminate HR or LR animals. A tendency (P = 0.06) to have lower plasma AMH was observed in heterozygous *FecG*^{E} (+/E) ewes. In conclusion, both AFC and plasma AMH can be used to select donor ewes with a higher potential of response for in vivo embryo production.

1. Introduction

In sheep embryo production programs, the variability of response after superovulation (SOV) is the most critical consideration [1]. In an attempt to overcome this issue, different methods have been proposed for screening the potential of the ewes' response to SOV. Recently, a pre-selection test based on a single high dose of equine chorionic gonadotropin (eCG), was suggested [2]. Since eCG is cheaper than FSH, this test consists of inducing SOV with a single dose of eCG, and the animals are classified as low or high responders, based on their corpus luteum (CL) count. Significant correlations between ovulation rates after eCG tests and subsequent FSH treatment were reported for Merino ewes and goats (Saanen does) [2,3]. In goats, the antral follicle count (AFC) was highly correlated with CL count and transferable embryos after SOV [3,4]. Therefore, AFC was considered a feasible tool for selecting

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goats with a greater likelihood of producing higher CL and embryo count after SOV. It is important to highlight that AFC can be easily and readily assessed through ultrasonography. However, in sheep, the association between AFC and the quality of response after SOV is not well-established and contradictory results have been reported [2,5,6].

In recent years, the assessment of plasma anti-Müllerian hormone (AMH) concentration has been proposed as a methodology for selecting better embryo donors in cattle and goats. In these species, higher AMH levels are associated with better performance in SOV and greater potential for embryo production [4,7,8]. In sheep, the AMH concentration at the beginning of the SOV treatment is highly correlated with the number of punctured follicles at laparoscopic ovum collection [9]. This evidence strongly suggest that AMH may also be used to select sheep with a higher potential for in vivo embryo production.

A series of mutations, mainly in the bone morphogenetic protein receptor 1B (*BMPR1B*), bone morphogenetic protein 15 (*BMP15*), and growth and differentiation factor 9 (*GDF9*) genes, have been reported to induce higher prolific rates in sheep [10]. Animals with mutations that affect prolificacy can show a superior response to SOV [11,12]. In Santa Ines ewes a mutation in the *GDF9* gene was found to affect the prolificacy and named $FecG^E$ [13]. Ewes homozygous for the $FecG^E$ mutation (E/E) showed an increased ovulation rate and twinning frequency. Data from the same study indicated an additive effect from the $FecG^E$ allele, with a tendency for heterozygous ewes (+/E) to present more twinning births than wild type (+/+), but less than E/E animals. Therefore, we hypothesized that Santa Ines ewes carrying the $FecG^E$ mutation could have a higher potential of response to SOV.

Despite these good prospects, the best practice for embryo donor selection is not well established. Thus, the present study aimed to assess the efficiency of different methods as a predictor of SOV response in ewes. These methods include the pre-selection eCG test, the AFC, the plasma AMH concentration, and the presence of the $FecG^E$ mutation.

2. Material and methods

This research was approved by the Ethical Committee for Animal Use of the Universidade Federal Fluminense (protocol 699/15), and conducted under the ethical principles of the Sociedade Brasileira de Ciência em Animais de Laboratório.

2.1. Experimental location, animals and study design

This study was performed during the breeding season, at Unidade de Pesquisa Experimental em Caprinos e Ovinos (UniPECO) in Cachoeiras de Macacu (22°27′45″ S latitude), Rio de Janeiro, Brazil. Twenty-five nulliparous Santa Ines ewe lambs were used, with an average age of 11.9 ± 1.1 months, a live weight of 38.8 ± 4.1 kg, and body condition score of 2.8 ± 0.3 (on a scale of 1 being emaciated, and 5 obese). Animals did not show any reproductive problems by clinical or ultrasonographic examination. Animals were housed in a confined area, and fed chopped Napier grass (*Pennissetum purpureum*) and a concentrate for their maintenance. Water and mineral salt (Salinas Ovinos, Salminas, Minas Gerais, Brazil) were provided ad libitum.

Initially, all animals were submitted to an eCG pre-selection test without being mated or inseminated. Twenty-one days later, an in vivo embryo production protocol was performed, with an FSH-based protocol. Ewes underwent antral follicle counting and evaluation of the AMH at two time points. Moreover, $FecG^E$ genotyping was performed.

2.2. Pre-selection test with eCG

Animals underwent SOV after a short-wave synchronization protocol according to the "Day 0 protocol concept" [14], adapted for Santa Ines sheep [15]. In brief, a sponge, impregnated with 60 mg of medroxyprogesterone acetate (MPA; Progespon, Schering Plough, São Paulo, Brazil), was inserted for six days. One day before sponge removal, 300 IU of eCG (Novormon, Schering Plough, São Paulo, Brazil) and 0.24 mg of cloprostenol sodium (Estron, Tecnopec, São Paulo, Brazil) were administered. Thirty-six hours after sponge removal, 0.025 mg of lecirelin (Gestran Plus, Tecnopec, São Paulo, Brazil) was administered. Eighty hours after sponge removal, 800 IU of eCG was administered and 60 h later, 0.24 mg of cloprostenol sodium was administered. Eight days after the eCG treatment, the CL count was assessed by laparoscopy, as previously described [2]. Based on the CL count, ewes were classified as having a high potential of response ($HR_{eCG} > 3$ CL) or low potential of response ($LR_{eCG} \leq 3$ CL), as previously suggested [2]. Immediately after laparoscopy, 0.24 mg of cloprostenol sodium was administered to induce CL regression (Fig. 1). All eCG used came from the same batch.

2.3. Counting the number of the follicles applied during the in vivo embryo production protocol

A short-wave synchronization protocol was performed, as described in the previous section. The SOV began 80 h after sponge removal, using 200 mg of FSH (Folltropin-V, Bioniche Animal Health, Ontario, Canada), administered in six decreasing doses, every 12 h (50/50, 30/30, 20/20 mg). At the time of the first FSH dose, a new sponge was inserted and not removed until the fifth dose. Together with the last FSH dose, 0.24 mg of cloprostenol sodium was administered, and 24 h later, 0.025 mg of lecirelin was administered. Ewes were mated with fertile Santa Ines rams every 12 h, between the last FSH dose and the end of the estrous behavior (Fig. 1). Six to seven days after the last mating, embryos were surgically recovered, as previously described [16], and the total number of recovered structures (including unfertilized oocytes, degenerated embryos, and viable embryos), the number of viable embryos, as well as the number of CL were recorded.

Ovaries of all ewes were scanned by the same operator using a portable transrectal B-mode ultrasonography equipment (Sonoscape S6, SonoScape, Shenzhen, China), with a 7.5 MHz linear transducer attached to a plastic support. Each ovary was located and fully scanned in a slow, continuous movement. Thereafter, using the cineloop function, all visible follicles (1–6 mm) were counted. Follicle counts were performed at two time points: $D0_{FSH}$ (immediately before insertion of the first sponge) and at $D9_{FSH}$ (immediately before the first FSH dose). Animals with <10 follicles were classified as having a low potential of response at both time points evaluated (LR_{AFC_D0FSH} and LR_{AFC_D9FSH}). Ewes with ≥10 follicles at $D0_{FSH}$ and at $D9_{FSH}$ were classified as having a high potential of response (HR_{AFC_D0FSH} and HR_{AFC_D9FSH}). The threshold values for AFC were determined using receiver-operating characteristic (ROC) analysis.

2.4. AMH assay

Two blood samples were collected for assessment of AMH. The first was collected immediately before the sponge insertion in the eCG trial protocol— that is, with animals at a random phase of the estrus cycle— ($D0_{eCG}$). The second was collected immediately before the first FSH dose, in the FSH SOV protocol— that is, with animals in a synchronized phase of the estrus cycle— ($D9_{FSH}$). Blood was collected by jugular venipuncture, using vacuum tubes

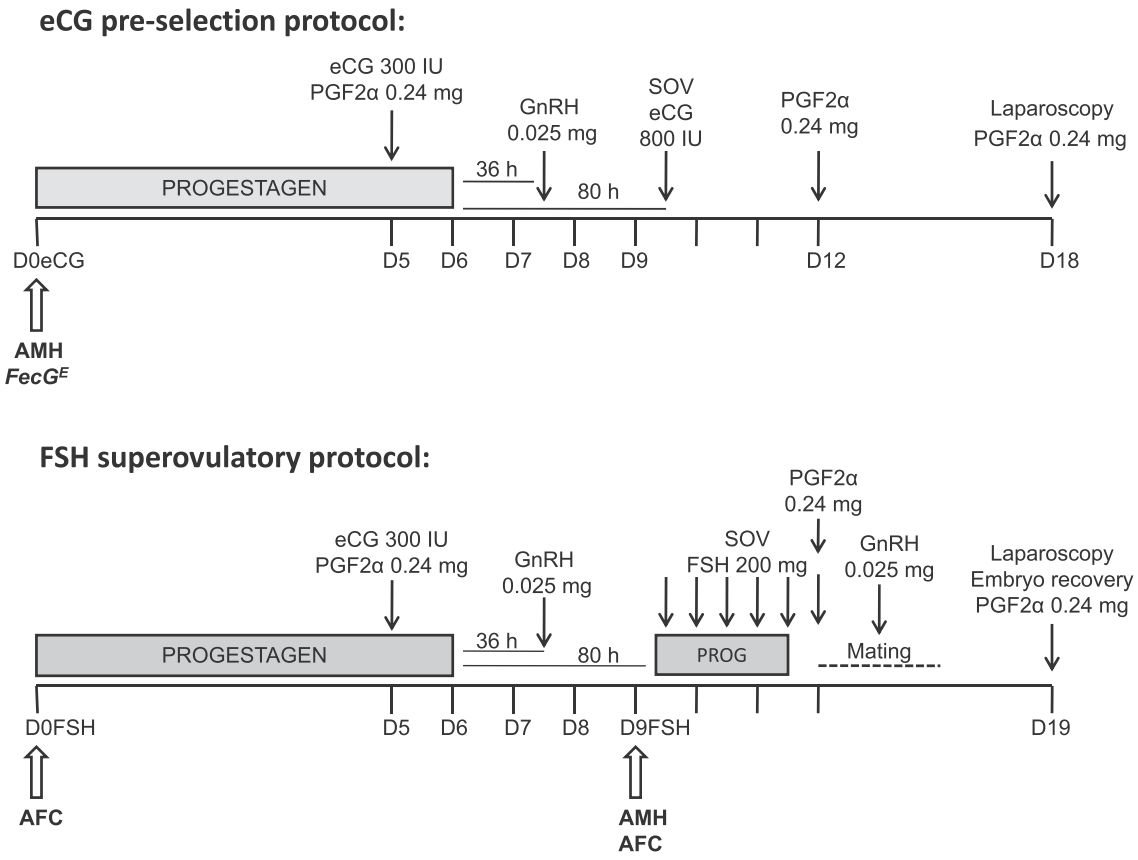

Fig. 1. Schematic representation of the eCG pre-selection test, FSH superovulation protocol, blood collections for AMH assessment, antral follicle count (AFC), and embryo recovery. A 21-day interval was applied between the end of the eCG test ($D18_{eCG}$) and the beginning of the FSH superovulation protocol ($D0_{FSH}$).

containing EDTA (Vacutainer, BD, Juiz de Fora, Brazil). Samples were immediately centrifuged at 1500*g* for 10 min, and plasma was removed and stored at − 20 °C until assay. Plasma AMH concentrations were measured by ELISA commercial KIT (Equine and Ovine AMH ELISA, AnshLabs, Texas, USA). Samples were evaluated as single samples, in a single assay. A calibration curve set was prepared with 5100 pg/mL for the maximum point and 20 pg/mL for the minimum. Samples were thawed at room temperature (23 °C), vortexed and centrifuged (3200*g* for 10 min). Then primary antibody was added, and samples were incubated overnight at 4 °C. After the secondary antibody addition, samples were incubated at room temperature for 90 min. These incubation periods were adopted to improve the sensitivity of the assay [17]. Test sensitivity was 9 pg/mL and intra-assay coefficients of variation ((SD/mean) × 100) were between 3% and 7%. Quality control was also considered, by including samples with known AMH concentrations. All data were found to be within the minimum and maximum points of the curve.

The threshold values for AMH were determined by ROC analysis. At $D0_{eCG,}$ ewes with plasma levels of AMH <177 pg/mL were classified as having a low potential of response (LR_{AMH_D0eCG}), and ewes with plasma levels of AMH ≥ 177 pg/mL were classified as having a high potential of response (HR_{AMH_D0eCG}). At $D9_{FSH,}$ ewes with <168 pg/mL of AMH were classified as having a low potential of response (LR_{AMH_D9FSH}), and ewes with plasma AMH ≥ 168 pg/mL as having a high potential of response (HR_{AMH_D9FSH}).

2.5. *FecG^E* genotyping

Genomic DNA was extracted from blood leukocytes using the salting-out method [18]. A *FecG^E* PCR-RFLP was carried out, amplifying the *GDF9* mature peptide region with the following primers (forward 5′-GACCAGGAGAGTGTCAGC; reverse 5′-CGACAGGTACACTTAGT). The PCR reactions were completed in 20 μl, with 0.5 μM of each primer, 1.5 mM $MgCl_2$, 400 μM dNTP mix, 1.5 U Taq polymerase (Invitrogen, São Paulo, Brazil) and 100 ng of genomic DNA. The PCR was conducted according to the following cycles: 93 °C/3 min; 35 cycles of 93 °C/40 s, 56 °C/40 s, 72 °C/40 s, and a final extension of 72 °C/5 min. Following this, 2 μL of the PCR reaction was digested overnight with TspRI (Biolabs, Massachusetts, USA), in accordance with the manufacturer's instructions. DNA fragments were differentiated within a 2.5% agarose gel, and genotyping was conducted according to the size of the digested DNA products. Considering that the *FecG^E* mutation has an additive effect [13], we hypothesized that ewes without this mutation (wild type: +/+) would be of low potential of response ($LR_{+/+}$) and the heterozygous ones (+/E) of high potential of response ($HR_{+/E}$).

2.6. Statistical analysis

Data analysis was completed using SAEG statistical software (SAEG 9.0, Universidade Federal de Viçosa, Minas Gerais, Brazil). The Lilliefors test was used to verify data normality. Parametric data were analyzed using a mixed model procedure, followed by Tukey's test, or Newman–Keuls multiple-comparison tests, for contrasting two or several means, respectively. The Pearson's correlation coefficient was used to assess correlations between the CL count after the FSH protocol and the CL count after the eCG protocol, AMH concentration and AFC. For all tests, $P < 0.05$ was considered statistically significant.

Table 1
Sensitivity, specificity, and positive and negative predictive values (PPV and NPV, respectively); accuracy and Kappa coefficient of different methods to select ewes with a high response to superovulation.

	CL eCG	AFC		AMH		$FecG^E$
	>3 CL	≥10 $D0_{FSH}$	≥10 $D9_{FSH}$	≥177 pg/mL $D0_{eCG}$	≥168 pg/mL $D9_{FSH}$	+/E
Sensitivity (%)	41.7	56	89	70	86	75
Specificity (%)	53.9	86	75	40	67	30.7
PPV (%)	45.5	91	67	82	50	50
NPV (%)	50.0	43	92	25	92	75
Accuracy (%)	48.0	64	80	64	72	44
Kappa	0.04	0.32	0.6	0.08	0.43	0.03

CL eCG, CL count at eCG pre-selection test; *AFC*, antral follicle count; *AMH*, plasma anti-Müllerian hormone; *+/E*, presence of one allele of the $FecG^E$ mutation. Thresholds used are indicated under each test abbreviation. $D0_{FSH}$ – first day of the protocol, just before sponge insertion; $D9_{FSH}$ – point of first FSH dose at superovulation; $D0_{eCG}$ – immediately before the beginning of the eCG pre-selection test, before any hormonal intervention.

As described in previous reports [17,19], data were subjected to ROC analysis, in order to determine AMH and AFC threshold values for selecting ewes with a higher response to superovulation treatment. In accordance with the gold standard for higher responders (ewes with CL > 7), each of their values for AMH, AFC and CL count (eCG protocol) were recorded, and used as a benchmark for positive (high response) and negative (low response) test outcomes in the FSH treatment. A comparison between the dichotomized test results and true individual status (as determined by their CL counts after the FSH protocol) allowed for an estimation of the diagnostic specificity (Sp = probability of a positive test outcome in a high-responding individual) and sensitivity (Se = probability of a negative test outcome in a low-responding individual). The diagnostic performance, in terms of Se (and 1 − Sp), were assessed for each possible AMH or AFC threshold value, using ROC analysis. The resulting pairs ((1−Sp), Se) were plotted on a unit square. Then, the area under the resulting ROC curve (AUC) was estimated using a non-parametric approach (Wilcoxon-area estimate) and compared with the expected value (AUC = 0.5) under the null hypothesis of a non-informative test, using the two sample Mann–Whitney rank-sum test [19]. When the AUC was significantly different from 0.5, the AMH or AFC threshold value was chosen, to maximize the Youden index ($J = Se + Sp - 1$), which corresponds to the point of the ROC curve closest to the upper left-hand corner of the unit square. The positive predictive value (PPV; denoted as %) was defined as the probability of a CL count after FSH protocol of >7 if the CL count at eCG protocol, AFC and AMH were above the threshold, and at the threshold for the $FecG^E$ mutation. The negative predictive value (NPV; denoted as %) was defined as the probability of the CL count after FSH protocol being ≤ 7 if the CL count after eCG protocol, AFC and AMH were below the threshold, and at the threshold for the $FecG^E$ mutation. Accuracy was defined as the proportion of agreement (correct answers) within the stipulated threshold. Finally, the kappa coefficient was used to assess the degree of agreement between the threshold set, and the CL count after the FSH protocol.

3. Results

3.1. Pre-selection eCG test

No significant correlations were found between the CL count after eCG treatment and the CL count, total number of recovered structures, or number of viable embryos after FSH protocol (r = −0.13; −0.09; −0.08, P > 0.05 respectively).

3.2. Number of antral follicles

Weak to moderately-positive correlations were found between AFC at $D0_{FSH}$ (first day of the FSH protocol, immediately before the first sponge insertion) and the CL count, total number of recovered structures and number of viable embryos after the FSH treatment (r = 0.45; 0.56 and 0.57, respectively, P < 0.05). Also, weak to moderately-positive correlations were found between AFC at $D9_{FSH}$ (the first FSH dose, follicular wave emergence) and the CL count, total number of recovered structures and number of viable embryos after the FSH treatment (r = 0.41; 0.39 and 0.42, respectively, P < 0.05).

Between the two time points ($D0_{FSH}$ and $D9_{FSH}$) at which AFC was determined, optimum diagnostic performance was observed at $D9_{FSH}$ (Table 1). Therefore, only results relating to AFC at $D9_{FSH}$ are shown (Fig. 2). In order to determine the AFC threshold values for selecting ewes with a high CL response (>7) to FSH treatment, AFC at $D9_{FSH}$ data were evaluated (Fig. 2). For each AFC taken as a possible threshold value, diagnostic sensitivity and specificity were determined (Fig. 2A). The area under the ROC curve was significantly different from the area corresponding to the null hypothesis

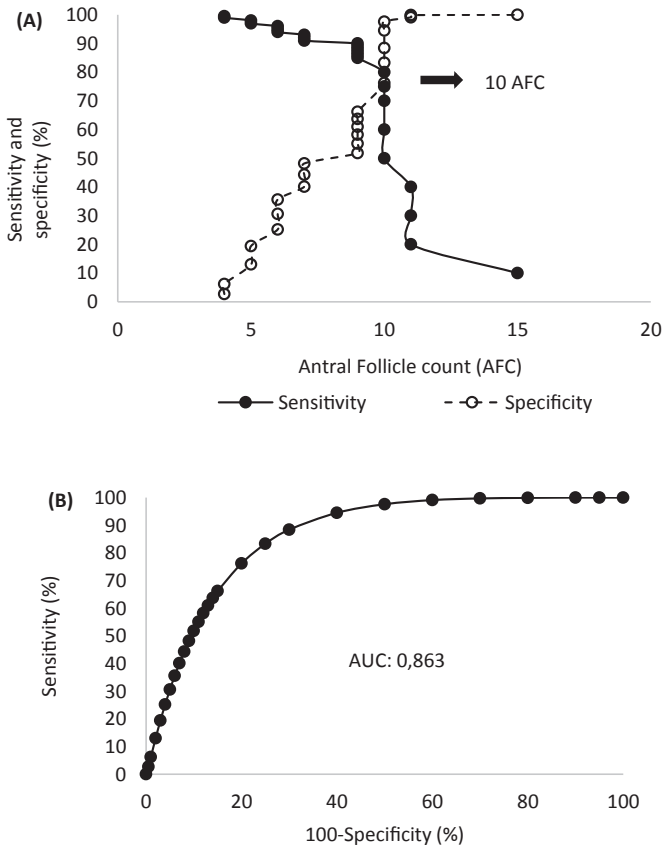

Fig. 2. Receiver-operating characteristic (ROC) analysis for determination of the antral follicle count (AFC) threshold for ewe lambs with >7 corpora lutea after the FSH protocol. Data were obtained from 25 ewe lambs. (A) Sensitivity and specificity curves. Each AFC was selected as a threshold for defining positive and negative test outcomes, ie, ewes with CL > 7 and ≤ 7, respectively, after the FSH protocol. Diagnostic specificity (Sp) was defined as the probability of a positive test outcome in an individual with CL > 7 after the FSH protocol. Diagnostic sensitivity (Se) was defined as the probability of a negative test outcome in an individual with CL ≤ 7 after the FSH protocol. Data for Se and Sp are expressed as percentages. (B) ROC plot representation of the data. The AFC threshold of 10 maximized the Youden index.

of a non-informative test ($P < 0.001$, Fig. 2B). The AFC at $D9_{FSH}$ which was found to maximize the Youden index was 10 follicles, corresponding to high PPV and NPV values.

3.3. Plasma AMH

The plasma AMH concentrations at $D0_{eCG}$ (immediately before the beginning of the eCG pre-selection test, and before any hormonal intervention) showed a weak correlation ($r = 0.34$; $P < 0.05$) with the total number of recovered structures after the FSH treatment. No significant correlations were found between AMH at the $D0_{eCG}$, and the CL count or the number of viable embryos after the FSH treatment. However, moderate to strong correlations were found between AMH concentrations at $D9_{FSH}$ (time of the first FSH dose) and the CL count, total number of recovered structures and number of viable embryos obtained after the FSH treatment ($r = 0.7$; 0.7 and 0.57, respectively, $P < 0.05$).

Between the two time points at which AMH was measured ($D0_{eCG}$ and $D9_{FSH}$), optimum diagnostic performance was observed in $D9_{FSH}$ (Table 1). For this reason, only results for AMH at $D9_{FSH}$ are shown (Fig. 3). Data for AMH recorded after an estrus synchronization protocol ($D9_{FSH}$) were evaluated, in order to determine the AMH threshold values for selecting ewes with a high CL response (>7) to FSH treatment (Fig. 3). For each AMH concentration taken as a possible threshold value, diagnostic sensitivity and specificity were determined (Fig. 3A). The area under the ROC curve was significantly different from the area corresponding to the null hypothesis of a non-informative test ($P < 0.001$, Fig. 3B). The plasma AMH concentration at $D9_{FSH}$ which was found to maximize the Youden index was 168 pg/mL, corresponding to high PPV and NPV values.

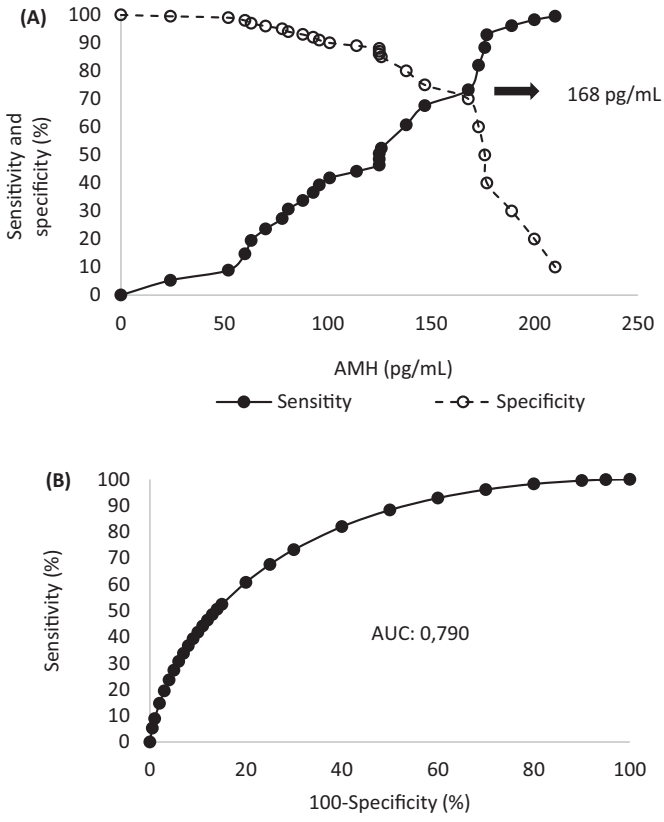

Fig. 3. Receiver-operating characteristic (ROC) analysis for determination of the AMH threshold for ewe lambs with >7 corpora lutea after the FSH protocol. Data were obtained from 25 ewe lambs. (A) Sensitivity and specificity curves. Each AMH concentration was selected as a threshold to define positive and negative test outcomes, ie, ewes with CL > 7 and ≤ 7 respectively, after the FSH protocol. Diagnostic specificity (Sp) was defined as the probability of a positive test outcome in an individual with CL > 7 after the FSH protocol. Diagnostic sensitivity (Se) was defined as the probability of a negative test outcome in an individual with CL ≤ 7 after the FSH protocol. Data for Se and Sp are expressed as percentages. (B) ROC plot representation of the data. The AMH threshold of 168 pg/mL maximized the Youden index.

Table 2
Data of FSH superovulation treatment (mean ± SD) from Santa Ines ewe lambs, wild type (+/+) or heterozygous (+/E) for the *FecG*^{E} mutation.

	Wild type (+/+; n = 7)	Heterozygous (+/E; n = 18)	Total
AMH (pg/mL)	149 ± 39*	109 ± 51*	120 ± 50
CL	8.1 ± 5.1	7.2 ± 4.8	7.5 ± 4.8
Rec Struc	7.4 ± 5.0	4.8 ± 3.9	5.6 ± 4.3
Viab Emb	4.6 ± 4.1	3.9 ± 3.1	4.1 ± 3.3

*P = 0.06. *AMH*, plasma anti-Müllerian hormone evaluated at the first FSH dose; *CL*, number of corpus luteum; *Rec Struc*, total number of recovered structures; *Viab Emb*, number of viable embryos.

3.4. The responses of the different genotypes

There was no difference between wild type (+/+) or heterozygous ewes (+/E) for the *FecG*^{E} mutation in terms of CL count (after FSH treatment), total number of recovered structures or number of viable embryos. Heterozygous ewes (+/E) tended to have lower plasma AMH values than wild ewes ($P = 0.06$) (Table 2).

3.5. Comparison of screening methods for embryo donors

Fig. 4 shows the post-FSH superovulation protocol responses of ewes classified as HR or LR based on the different methods evaluated. Table 1 shows the sensitivity and specificity values, positive and negative predictive values, and the accuracy and Kappa coefficient of the different methods used for screening donors for their potential for in vivo embryo production.

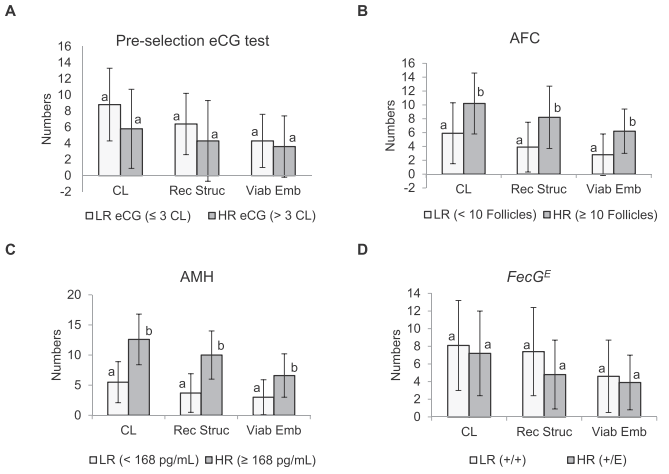

Fig. 4. Comparison of four screening methods used to classify Santa Ines ewe lambs as having low potential of response (LR) or high potential of response (HR) for in vivo embryo production. The screening methods were: **A**– CL count after a pre-selection eCG test (eCG); **B**– antral follicle count, evaluated at the first FSH dose (AFC); **C**– plasma anti-Müllerian hormone, evaluated at administration of the first FSH dose (AMH); **D**– presence of one allele (+/E), or absence of the *FecG*^{E} mutation (+/+). Thresholds used for each screening method are indicated. CL count, total number of recovered structures (Rec Struc) and number of viable embryos (Viab Emb) after an FSH-based superovulation are presented.

4. Discussion

The results indicated that both AFC and plasma AMH provide accurate methods for selecting sheep with higher potential to MOET (multiple ovulation and embryo transfer). However, these screening methods showed different results at different time points. The optimal moment for AFC and AMH measurement was after the estrus synchronization protocol ($D9_{FSH}$), and at the emergence of a new follicular wave [14]. After estrus synchronization protocol, the population of small antral follicles is expected to be larger [14], if no influence exists from large follicles or active CL. Additionally, in mammals, AMH production is higher in small, growing follicles [20]. Therefore, $D9_{FSH}$ may be more favorable for AFC and AMH evaluations.

The eCG pre-selection test could not effectively identify ewes with higher potential of response to MOET. Contradictory results have been reported in sheep and goats [2,3]. In our study, ewes underwent SOV at the beginning of a new follicular wave, using the Day 0 protocol [14]. This protocol can optimize the response to SOV, primarily by eliminating the negative effect of large follicles at the beginning of SOV [1,14]. However, even with application of this concept, the positive results previously reported for the eCG test were not repeated under our experimental conditions. Therefore, further investigations evaluating more hormonal profiles and ovary activity should be performed, in order to explain why and under what circumstances the eCG test can show low efficiency.

The presence of one allele of the $FecG^E$ mutation did not improve embryo production. Moreover, the different genotypes (+/+ or +/E) did not influence the response to the FSH treatment. Silva et al. [13] reported higher ovulation rates and twinning frequency in sheep homozygous for the $FecG^E$ allele (E/E). It is expected that these animals have higher numbers of recruited follicles and also a lower atresia rate, leading to a higher response to the FSH- based superovulation regime. The E/E genotype is found in only 3.9% of ewes in flocks that are not selected for prolificacy, and none of the experimental animals showed this genotype. However, one interesting finding was the tendency ($P = 0.06$) for lower AMH levels in +/E ewes. The *GDF9*, together with other proteins from the *TGF-β* (transforming growth factor-β) superfamily, is known to be an important intraovarian regulator [21]. Moreover, homozygous ewes for the $FecB^B$ allele (Booroola mutation) showed lower concentrations of AMH, probably due to lower *BMP* (bone morphogenetic proteins) activity in the ovary. This may occur due to partial loss-of-function in the *BMPR1B* receptor [22]. In this way, lower AMH levels in $FecG^E$ ewes may be attributed to lower *GDF9* activity in the granulosa cells, which affects AMH secretion. More detailed investigations should be performed to elucidate the association between $FecG^E$ mutation and AMH secretion.

The AFC at $D0_{FSH}$ and at $D9_{FSH}$ showed weak to moderately-positive correlations with the CL count, total number of recovered structures and number of viable embryos after FSH treatment. In cows, the AFC is positively correlated with the total number of recovered embryos [23,24] and in goats, AFC is positively associated with CL count, in response to SOV [3]. However, in sheep, AFC remains controversial as a screening method for better donors. A recent publication found no significant correlation between AFC and embryo production [2]. Even in studies that reported significant correlations between AFC and subsequent SOV performance, the results were unremarkable [25,26]. Differences in the features of the ultrasound unit or practical skill may lead to imprecise AFC measurements and limit the application of this technique. Unexpectedly, despite the weak to moderate correlation found, AFC at the $D9_{FSH}$ could effectively segregate HR_{AFC_D9FSH} from LR_{AFC_D9FSH} (Fig. 2B). Ewes with high AFC (evaluated at the emergence of the first follicular wave) showed a better response to SOV, than ewes with low AFC [27]. Therefore, despite the limitations, AFC performed through ultrasonography could be used to select more productive embryo donors.

It is important to note the strong associations ($P < 0.05$) between the plasma AMH levels at $D9_{FSH}$, and the CL count and the total number of recovered structures. Similar results have been reported in cows (AMH vs CL after SOV) and goats (AMH vs CL and AMH vs collected embryos, both after SOV) [4,7]. Our findings confirm that plasma AMH levels can be used to select ewes with higher potential of response to in vivo embryo production. One great advantage of AMH measurement is that it is highly reproducible, making it possible to select better donors with a single evaluation [7,20]. The present results also show that, in Santa Ines ewes, AMH measurement should be performed after an estrus synchronization protocol, to improve its efficiency.

When considering the CL count, total number of recovered structures, and number of viable embryos from ewes classified as HR or LR by the different methods (Fig. 4A, B, C, and D), only AFC and AMH (both evaluated at $D9_{FSH}$) could efficiently identify ewes with higher potential of response. These findings have an important field application. Selecting only HR ewes for the SOV protocol could significantly increase or even double the number of embryos produced (Fig. 2B and C).

A meta-analysis review, addressing the use of AMH in assisted reproductive technology for humans, showed that AMH serum levels could identify poor responders, with sensitivity and specificity ranging from 0.40 to 0.97, and 0.41 to 1.00, respectively. According to these authors, similar efficiency can be achieved using AFC. Additionally, in women with excessive ovarian response, AMH sensitivity and specificity ranges were between 0.57 and 0.93, and 0.62 and 0.96, respectively [28]. In our study, it was noted that both AFC and AMH (both at $D9_{FSH}$) are reliable in identifying ewes with poor response to SOV, indicated by high values of sensitivity and NPV. Also, at $D9_{FSH}$, AFC and AMH methods showed good and moderate agreement, respectively (Kappa coefficient), high accuracy, and satisfactory specificity (Table 1). This confirmed the suitability of these screening tests for segregating HR and LR ewes. It should be stressed that higher MOET efficiency can greatly expand the application of this biotechnology, accelerate genetic improvement of commercial herds, favor the preservation of endangered species, and positively contribute to related research lines.

5. Conclusions

Ewes with higher potential for in vivo embryo production can be selected by AFC or plasma AMH measurement, after an estrus synchronization protocol. Under our experimental conditions, the eCG pre-selection test and genotypes for the $FecG^E$ mutation (heterozygous or wild type) could not identify high-responding or low-responding ewes.

Declaration of interest

The authors declare that there is no conflict of interest.

Authors' contributions

PHNP: conception and study design, data collection, statistics and creation of the first draft. MFAB: study design, statistics and data collection. LSR, GMB, CRL, and KMS: data collection. EKNA and JMGSF: critical evaluation of the manuscript. JFF and FZB: elaboration of the hypothesis, experimental design and data collection. In addition, all authors contributed to the writing, revision, and approval of the final version of the manuscript.

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