Theriogenology 84 (2015) 784–790

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Preselection of high and low ovulatory responders in sheep multiple ovulation and embryo transfer programs

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ARTICLE INFO

Article history:
Received 3 October 2014
Received in revised form 1 May 2015
Accepted 12 May 2015

Keywords:
Multiple ovulation
Embryo recovery
Follicular population
Multiple ovulation and embryo transfer

ABSTRACT

The present study evaluated the feasibility of carrying out an easy-to-handle and cost-efficient test for the preselection of high– and low–ovulatory responder ewes under superovulatory protocols. The test was based on the assessment of the number of ovulations obtained in response to the administration of a single-shot eCG treatment. The predictive value of the test was determined by comparing the number of ovulations with yields obtained in response to a multiple-dose FSH treatment. In addition, the study determined possible effects of follicular status at first FSH dose and their relationship with subsequent ovarian response. A total of 31 Merino ewes received hormonal treatment comprising the administration of 800 IU of eCG at the end of progestative treatment. Twenty-three days later, multiple-dose FSH treatment (80-mg FSH, in six decreasing doses between Days 12 and 14 of a second progestative treatment) was applied to the same ewes. The study showed a significant relationship between the number of corpora lutea obtained in response to eCG treatment with respect to those obtained in response to FSH treatment ($r = 0.791$; $P < 0.05$), which resulted in 84% recurrence rate. The number of embryos was greater for high-responder in relation to low-responder ewes (7.2 ± 3.7 and 4.0 ± 3.9, respectively; $P < 0.05$), whereas rates of recovery and fertilization were similar between groups ($P > 0.05$). Hence, there was a tendency for a higher mean of grades 1 and 2 embryos in high-responder in relation to low-responder ewes (6.1 ± 3.8 and 3.7 ± 4.0, respectively; $P < 0.1$). No significant relationship was found between the number of corpora lutea in response to FSH treatment and the number of small and total follicles at first FSH dose ($P > 0.05$). However, a negative low relationship was found between the presence of large follicles and the ovulation rate in response to FSH treatment ($r = -0.361$; $P < 0.05$). In conclusion, the results show the feasibility of carrying out an easy-to-handle and cost-efficient procedure for the preselection of embryo donors. The procedure was based on high recurrence rate between hormonal treatments, which in turn accounts for a distinctive ewe ovulatory response.

1. Introduction

Multiple ovulation and embryo transfer (MOET), though to a much lesser extent than in other species such as bovine, is applied in ovine to increase the progeny from selected ewes [1–4]. On the one hand, a MOET protocol consists of the administration of gonadotropin treatment to

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0093-691X/$ – see front matter © 2015 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2015.05.011

substantially increase the number of ovulations, and therefore embryos, during a synchronized estrous cycle in high-merit sheep; and on the other hand, it consists of both the recovery and transfer of such embryos to recipient sheep.

However, regardless of the various advances [4,5] in the control of extrinsic (source and purity of hormones, and protocols of administration) and intrinsic factors (breed, age, nutritional and reproductive status), the efficiency of MOET protocols still remains hampered by a high individual variability in the ovulation rate and in the number of recovered embryos. Thus, the implementation of embryo transfer programs under field conditions has been limited [6], even when applying the same superovulatory treatment to individuals of the same breed and flock.

The intense research activities developed during the past decade pointed to a prominent role of the ovarian status at the beginning of superovulatory treatment [7–11]; hence, different protocols were designed to induce the optimal ovarian status before superovulation [12,13]. In spite of that, a high interindividual variability in response to exogenous ovarian stimulation is still considered the major factor limiting the success of MOET programs in sheep. Moreover, the existence of a high intraindividual repeatability in response to successive superovulatory treatments has also been established [14–16], which has been hypothesized to be related to the primordial follicle pool intrinsic to each ewe (i.e., the ovarian reserve) [17], and consequently, related to follicular development [18]. Therefore, the differentiation of ewes showing high or low response to superovulatory treatments would be extremely useful in MOET programs.

These facts suggest the possibility of applying predictive tools for preselection of ewes with high ovulatory response, which is an issue of critical importance in embryo programs for ethical, technical, and economic reasons. A practical, ethical, and cost-efficient approach for such preselection consists of performing an exogenous FSH ovarian reserve test, based on the administration of a single-shot treatment and on the subsequent follicular development evaluation [17]. The use of a single eCG dose has been reported as a successful tool to differentiate populations of prolific carriers from populations of nonprolific carriers in adult ewes [19,20] and in prepubertal ewe lambs [21–23]. In spite of that, this treatment does not enable individual genotype differentiation [19,21,23] because of a large variability in the ovulation rate within ewes of the same genotype [19]. However, there are not any systematic studies determining the applicability and predictive value of such strategies in superovulatory protocols under field practice.

Thus, the aim of the study was to evaluate the feasibility of establishing a preselection test to differentiate between ewes as high or low responders before the application of a multiple ovulation and embryo recovery (MOER) protocol. This test was based on the administration of a single-shot eCG treatment and on the assessment of the number of ovulations by minimally invasive procedures. The predictive value of the test was determined by comparing the number of ovulations with yields obtained in response to multiple-dose FSH treatment initiated 23 days later. Concomitantly, the study also assessed the effect of follicular status at the beginning of FSH administration and its relationship with MOER yields.

2. Materials and methods

The study was conducted at the Instituto Nacional de Tecnología Agropecuaria located in San Carlos de Bariloche (Río Negro, Argentina) at 41° S latitude. Ethical concerns were taken into account by adhering to local animal welfare regulations and practices.

Thirty-one healthy multiparous 4-year-old Merino sheep, in good body condition (>3 out of five; scale from 0, emaciated, to 5, obese) and moderate body weight (45.6 ± 2.2 kg), were used during the breeding season (April–May 2011). Ewes had lambed at least 5 months before and weaned 2 months before the superovulatory treatment. Animals were kept outdoors in a sheltered pen under natural day length and were fed a live-weight maintenance ration. Water was provided *ad libitum*.

2.1. Preselection test procedure

The test carried out to classify high and low ovulatory responders consisted of the insertion of a progestagen-impregnated intravaginal pessary (60 mg of medroxyprogesterone acetate, Progespon; Syntex, Argentina) for 14 days and an intramuscular (IM) administration of 800 IU of eCG (Novormon, Syntex) at the time of pessary removal. Estrus detection was performed twice daily (8 AM and 8 PM) from 24 to 72 hours after pessary removal with vasectomized adult rams (Day 0 = day of estrus). On Day 4 of the estrous cycle after eCG treatment, the number of corpora lutea (CL) was determined by laparoscopy. Immediately afterward, an IM 125-μg dose of cloprostenol (Estrumate; Intervet/Schering-Plough, Argentina) was administered to induce CL regression.

Ewes were deprived of food for 24 hours and water for 12 hours before laparoscopic intervention. Laparoscopies were carried out by placing ewes in dorsal recumbent position on a standard cradle for laparoscopic procedures. The surgical field, cranial to the udder, was shaved and disinfected. The procedure was carried out under IM general anesthetic (0.05 mL/kg of acepromazine maleate, Peacefully 1%; Vabriela, Buenos Aires, Argentina). Subcutaneous local anesthetic (0.05 mL/kg of lidocaine, Frankaina 2%; Fatro Von Franken, Buenos Aires, Argentina) was injected at the trocar insertion. A pneumoperitoneum was induced with a Veress needle (Endopath; Ethicon Endo-Surgery, Cincinnati, OH, USA; 2-mm diameter; 120-mm length) placed in the midline. The needle was attached to an automatic insufflator (Richard Wolf Medical Instrument Corp., Rosemont, IL, USA) by a flexible gas hose to enable approximately 2 L of inert air into the abdominal cavity. Afterward, to visualize the ovaries, an endoscope (Richard Wolf, Knittlingen, Germany; 4-mm diameter; 170-mm length) was inserted into the abdominal cavity through a trocar, approximately 5-cm cranial to the udder and 5 cm to the left side of the midline. The ovaries were handled by using the Veress needle (Endopath; Ethicon Endo-Surgery).

Assessment of the ovarian structures (CL and follicles) was always performed by the same operator. Images of the ovaries were recorded and backed up with a digital camera.

Once the laparoscopic procedure was completed, each ovary was gently flushed through the Veress needle using physiological saline solution with heparin (0.05 mg/mL of heparin; Nortia, Buenos Aires, Argentina) at 38 °C to prevent possible adhesions. Finally, trocar wounds were treated with a local antibiotic-cicatrizing solution (Young Plata; Quimagro, Buenos Aires, Argentina).

2.2. MOER procedure

Five days after prostaglandin administration, a second progestagen-impregnated intravaginal pessary was inserted (Day 0 = day of second pessary insertion) for 14 days. The presence of follicles of 2 mm or greater was determined by laparoscopy before the beginning of the superovulatory treatment, considering the number of small (2–4 mm) and large (>4 mm) follicles on the ovarian surface in each ewe. Follicle diameter was measured by means of a palpation probe with centimeter marks (Karl Storz Veterinary Endoscopy-America, Inc, Goleta, CA, USA; 3-mm diameter; 200-mm length) but carved to millimeter marks in one section. The palpation probe was introduced into the abdominal cavity through a 5-mm incision.

The superovulatory treatment, adapted from Gibbons et al. [24], consisted of the administration of 80-mg FSH (Folltropin-V; Bioniche, Canada) in six decreasing IM doses (18 mg × 2; 14 mg × 2; 8 mg × 2) administered twice daily from the morning of Day 12 to 12 hours after pessary removal. A single IM dose of 200 IU of eCG (Novormon; Syntex) was administered with the fifth FSH dose immediately after pessary removal.

Estrus detection was performed twice daily (8 AM and 8 PM) from 24 to 72 hours after pessary removal, with vasectomized adult rams. Twelve hours after estrus detection, ewes were artificially inseminated with 100 million frozen–thawed spermatozoa from the same batch and ram (0.25-mL dose; Triladyl + 20% egg yolk; thawing at 37 °C; postthaw progressive forward motility, 40%–45%; artificial insemination within 15 minutes after thawing). Half of each semen dose was introduced into each uterine horn by the laparoscopic method [25], using a cannula for intrauterine artificial insemination (Aspic for ewe insemination, 23-ga needle; IMV, L'Aigle, France).

On Day 8 after pessary removal, the number of ovulations was recorded by laparoscopic procedure and, immediately afterward, embryos were surgically recovered under general anesthesia. Ewes, deprived of food and water for 24 hours, were IM administered with xylazine (2 mg/10 kg of Kensol 2%; Konig, Buenos Aires, Argentina) and ketamine hydrochloride (25 mg/10 kg of Ketalar; Parke-Davis, Buenos Aires, Argentina). Furthermore, local anesthesia was administered in the surgical field (0.05 mL/kg of lidocaine; Frankaina 2%, Fatro Von Franken). Embryos were collected by laparotomy [1], performed by the same operator, flushing each uterine horn with 20 mL of commercial embryo recovery medium (Vigro Complete Flush; Bioniche, USA), prewarmed to 38 °C, and supplemented with 10% adult bovine serum (Internegocios, Buenos Aires, Argentina). Embryo recovery medium was injected by a sterile syringe with an 18-ga blunt needle, inserted close to the uterine horn bifurcation and directed from the uterine horn toward the uterotubal junction, where a catheter was attached with silk (3/0, Ethicon,

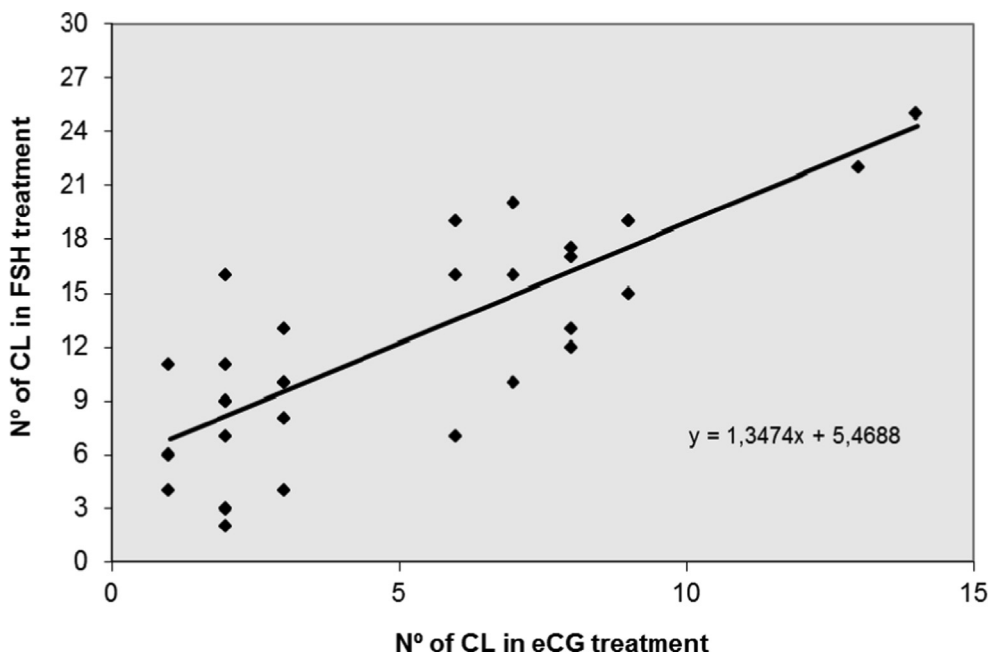

Fig. 1. Relationship between the number of corpora lutea (CL) in response to eCG and FSH treatments.

Table 1
Correlation (*r* value) of corpora lutea number in response to eCG (eCG CL) and FSH (FSH CL) treatments and the number of oocytes and embryos, number of embryos, and number of embryos of grades 1 and 2.

Embryo yields	eCG CL	FSH CL
No. of oocytes and embryos	0.618[a]	0.736[a]
No. of embryos	0.472[a]	0.615[a]
No. of grades 1 and 2 embryos	0.342[b]	0.479[a]

[a] Indicates significant differences ($P < 0.05$).
[b] Indicates tendency toward statistical significance ($P < 0.1$).

Brazil). The catheter consisted of a pediatric nasogastric tube (k33) fastened to a blunt needle (50/20), with a central opening at the tip and two lateral openings. Recovered embryos were examined under a stereomicroscope (Olympus SZ; Olympus Optical Co., Ltd., Tokyo, Japan) at 20 to 40 × magnification and classified according to the International Embryo Transfer Society criteria [26]. Only embryos in morula and blastocyst stages classified as grade 1 (excellent) or grade 2 (good) were considered transferable embryos.

Immediately after embryo recovery, a single IM injection of 125-μg cloprostenol (Estrumate; Intervet/Schering-Plough) was administered to induce CL regression.

2.3. Indexes of superovulatory response

The following information was recorded for each ewe: number of CL, number of oocytes, number of embryos (grades: 1–4), and number of embryos of grades 1 and 2. The rate of oocytes and embryos was calculated, for each ewe, by dividing the number of oocytes plus embryos by the number of CL. The rate of embryo recovery was calculated, for each ewe, by dividing the number of embryos by the number of CL. The rate of embryos of grades 1 and 2 was calculated, for each ewe, by dividing the number of grade 1 plus 2 embryos by the number of embryos. Fertilization rate was calculated, for each ewe, by dividing the number of embryos by the number of oocytes plus embryos. Rates were expressed as percentages.

Ewes were grouped into high or low ovulatory responders to eCG (high, >3 CL; low, ≤3 CL) and to FSH treatments (high, >12 CL; low, ≤12 CL) to evaluate the recurrence rate between eCG and FSH treatments. A CL cutoff was used to divide the total number of ewes into even ovulatory responder groups in both hormonal treatments.

2.4. Statistical analysis

Analysis of data was performed using Statistical Analysis System software package [27]. Simple linear regression analysis was performed to assess the relationship between the number of small, large, and total follicles at first FSH dose and the number of CL in response to FSH treatment; and between the number of CL in response to eCG treatment and the number of CL in response to FSH treatment. Analysis of variance was used to compare embryo production and rates of recovery and fertilization between high– and low–ovulatory responder ewes to FSH

Table 2
Ovarian response and embryo production (mean ± standard deviation) in high– and low–ovulatory responder Merino ewes subjected to multiple-dose FSH superovulatory treatment.

Ovulatory responder ewes	Ewe donors[e]	Corpora lutea		Oocytes and embryos		Embryos			Oocytes		Fertilization rate	Grades 1 and 2 embryos[f]		
	n	n	Mean	n	Mean	%	n	Mean	%	n	Mean	%	n	Mean	%
High	15	265	16.7 ± 4.6[a]	146	9.1 ± 4.0[a]	56.3 ± 20.9	115	7.2 ± 3.7[a]	47.3 ± 32.2	31	1.9 ± 2.8[c]	81.5 ± 26.4	97	6.1 ± 3.8[c]	87.8 ± 30.4
Low	16	123	8.2 ± 3.9[b]	66	4.4 ± 3.8[b]	52.3 ± 31.2	60	4.0 ± 3.9[b]	44.0 ± 21.3	6	0.4 ± 1.3[d]	91.7 ± 23.6	56	3.7 ± 4.0[d]	87.5 ± 29.8

[a,b]Different letters within columns indicate significant differences ($P < 0.05$).
[c,d]Different letters within columns indicate tendency toward statistical significance ($P < 0.1$).
[e] Ewes that exhibited estrus.
[f] Grades 1 and 2 [26].

treatment. Statistical analysis of results expressed as percentages (rates of recovery and fertilization) was performed after transforming each ewe percentage to the arcsine square root. The analysis also tested the correlations between the number of CL obtained in response to eCG and FSH treatments with respect to the number of oocytes and embryos, the number of embryos, and the number of embryos of grades 1 and 2. Results were expressed as the mean ± standard deviation. Statistical significance was accepted from $P < 0.05$.

3. Results

The assessment of follicular population at first FSH dose showed a mean of 13.1 ± 5.8 small follicles (range: 0–23), 1.3 ± 1.0 large follicles (range: 0–4), and 14.4 ± 5.6 total follicles (range: 3–24). There was not any relationship between the number of small follicles at first FSH dose and the number of CL in response to FSH treatment, or the number of total follicles and the number of CL. However, the number of large follicles was negatively correlated with the number of CL ($r = -0.361$; $P < 0.05$).

On the basis of ovarian examination in response to eCG treatment, a mean of 5.3 ± 3.5 CL was calculated per ewe. However, the mean doubled (12.6 ± 6.0 CL) per ewe at ovarian examination in response to FSH treatment.

As shown in Figure 1, the assessment of responses to eCG and FSH treatments in the same ewe indicated a significant positive relationship ($r = 0.791$) between the number of CL obtained in response to each treatment ($P < 0.05$).

Correlations between embryo yields and the number of CL in response to both hormonal treatments are shown in Table 1. On the basis of analysis of the number of CL in response to eCG, significant positive correlations were observed with the number of oocytes and embryos and with the number of embryos ($P < 0.05$). The analysis also showed a tendency toward significance with the number of embryos of grades 1 and 2 ($P < 0.1$). Additionally, on the basis of analysis of the number of CL in response to FSH treatment, significant positive correlations were observed with the number of oocytes and embryos, with the number of embryos, and with the number of embryos of grades 1 and 2 ($P < 0.05$).

A total of 175 embryos were recovered from the 31 ewes. High number of embryos of grades 1 and 2 (62 morulae and 91 blastocysts) was obtained due to the recovery of low number of embryos of grades 3 and 4 (~13%).

Once ewes were grouped into high or low ovulatory responders, the mean of CL obtained in response to eCG treatment was 8.3 ± 2.3 in high responders ($n = 16$) and 2.1 ± 0.7 in low responders ($n = 15$). The mean of CL in response to FSH treatment was 16.7 ± 4.6 in high responders ($n = 15$) and 8.2 ± 3.9 in low responders ($n = 16$).

In the present study, the recurrence rate was defined as the ovarian response after FSH administration in relation to prior response to eCG treatment. Thirteen ewes of 16 with high ovulatory response to eCG treatment still showed high ovulatory response after FSH treatment (81%). Thirteen ewes of 15 with low ovulatory response to eCG treatment still showed low ovulatory response after FSH treatment (87%). All in all, 26 ewes of 31 still showed high or low ovulatory response after FSH treatment (84%).

As noted in Table 2, when comparing superovulatory response indexes, there were significant differences in the number of oocytes and embryos and in the number of embryos between high and low ovulatory responders to FSH treatment ($P < 0.05$). In addition, the analysis showed that the mean of grades 1 and 2 embryos and the mean of oocytes tended to be greater in high responders than in low responders ($P < 0.1$). In contrast, the mean of grades 3 and 4 embryos did not differ between groups (low: 0.27 ± 0.6 vs. high: 1.13 ± 2.8; $P > 0.05$). Both recovery and fertilization rates lacked significant differences between high and low responders ($P > 0.05$). The rate of grades 1 and 2 embryos in relation to the number of oocytes and embryos also lacked significant differences between groups (low: 79.2 ± 34.9% vs. high: 73.3 ± 34.6%; $P > 0.05$).

4. Discussion

The results of the present study show the feasibility of implementing an easy-to-handle and cost-efficient procedure to differentiate between high- and low-responder ewes under field conditions, by applying a single-shot eCG treatment before the application of MOER protocols. At the same time, as observed in previous literature [15,28], it is noteworthy that superovulatory response and rates of fertilization were not affected by prolonged exposure to progesterone and exogenous gonadotropin treatments.

When considering animal welfare, emphasis should be placed on the use of nonsurgical procedures. In the present study, laparoscopic technique was applied to determine the exact number of CL, which would not have been possible by ultrasonography. In this regard, ultrasonography constitutes a noninvasive alternative to examine luteal tissue and can be used to determine whether a ewe has ovulated or multiovulated, thus avoiding laparoscopic procedure [29].

High correlation coefficient ($r = 0.791$) and high recurrence rate (84%) allow to differentiate, with great accuracy, between high- and low-responder ewes. In the same way, significant positive correlations observed between the number of CL in response to eCG and FSH treatments and the number of embryos and number of grades 1 and 2 embryos indicate that the single-shot eCG test was useful to identify not only ewes with higher ovulation rate but also ewes with larger number of total and transferable embryos. In fact, previous studies in prepubertal heifers [30] showed consistent relationships between the number of follicles and the number of aspirated and usable oocytes in successive stimulations of high and low responders. This evidence supports the hypothesis that ewe genotype plays a primary role in establishing follicular recruitment and developmental capability of oocytes, and thus influencing superovulatory yields [14].

Once ewes were grouped into high or low ovulatory responders to FSH treatment, numbers of total and transferable embryos in low-responder ewes (4.0 and 3.7, respectively) almost doubled in high-responder ewes (7.2 and 6.1, respectively).

Rates of embryo recovery in the present study are lower than those cited in literature but similar to rates obtained in

previous studies from the same authors [15,31]. Regarding high fertilization failure observed in Merino ewes in comparison with other breeds when practicing cervical insemination [32], laparoscopic technique was the alternative to carry out artificial insemination, although this technique reduces recovery rates [33].

The analysis of ovarian follicular population at first FSH dose showed a high interindividual variability, as described by other authors [34]. Reasons for this variability are still unknown. It could be caused by intrinsic factors, such as the number of primordial follicles at birth or genetic mechanisms, or by external factors, such as nutrition or environmental conditions [34]. In contrast with previous literature [34–36], the present study did not show significant relationship between numbers of small and total follicles at first FSH dose and the number of CL in response to FSH treatment. Differences between current and previous findings could be attributed to different experimental procedures, such as hormonal superovulation protocol or follicle size classification. This evidence also sheds light on the need for further specific studies to evaluate the precise role of ovarian status in response to assisted reproductive technologies. However, significant low relationship was found between the number of large follicles (>4 mm in size) at first FSH dose and the number of CL in response to FSH treatment. Previous studies did not show any significant correlation between the number of 4- to 5-mm follicles and superovulatory response indexes [35,36]. On the basis of present and previous [37,38] studies, the largest follicle of each wave has a limited direct effect on the growth of small follicles at gonadotropin-dependent stages in sheep, unlike in cattle. These follicles are able to respond to exogenous FSH, both preventing early atresia and increasing growth rate from small to ovulatory follicles, which are mechanisms responsible for increased ovulation rates. Hence, the presence of large follicles does not prevent ovulation of small follicles because they still retain their capability to respond to exogenous gonadotropin stimuli and to resume growth [6]. However, embryo yields are ultimately affected by a reduction in the embryo recovery rate rather than in the ovulation rate [36] due to inadequate follicular development and disturbances in ovulation [39] or to reduced developmental competence of oocytes [40].

In conclusion, high recurrence rate in ovarian response between eCG and FSH treatments allows the selection of high-genetic-merit ewes with predictable high embryo yields. This selection is carried out by choosing donors with high ovarian response to a cost-efficient eCG treatment. The recurrence rate evidenced in this article is expected to reduce costs in MOET programs by avoiding treatment and surgical embryo recovery in ewes identified as low responders. The nonsignificant or low relationship between different diameter follicle populations at first FSH dose and subsequent ovarian response reinforces the hypothesis that each ewe has a strong intrinsic or individual factor determining their own degree of response to superovulatory treatments.

Acknowledgments

This study was supported by funds from the Instituto Nacional de Tecnología Agropecuaria (Project AESA 203930). The authors are grateful to Nicolás Giovannini for his participation in the statistical analysis of data.

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