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In vivo embryo production and recovery in lacaune ewes after imposing a superovulation treatment regimen is related to pFSH dose

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ABSTRACT

This study was conducted to assess effects of different doses of pFSH on follicular recruitment, superovulatory response, ova/embryo recovery, and embryo yield in lactating ewes. Ewes ($n = 24$) had a superovulation treatment regimen imposed. All ewes were implanted with a progesterone intravaginal device for 9 d, and administered either 100 (G-100) or 200 (G-200) mg pFSH, proportioned into six doses administered at 12-h intervals, starting 60 h before device removal. At 7 days subsequent to progesterone device removal, there were non-surgical embryo recoveries (NSER) from ewes having three or more corpora lutea. At the time of the first pFSH injection, number of antral follicles were similar ($P < 0.05$) between ewes in the G-100 and G-200 group, however, there were more 3.1–4.0 mm follicles in ewes of the G-200 than G-100 group at the time of the second pFSH administration. Estrous response and CL number were less ($P < 0.05$) in ewes of the G-100 (66.7 % and 2.6 ± 0.7) than G-200 (91.7 % and 11.6 ± 1.2) group. There were embryo collections from 100 % and 90.9 % of ewes in the G-100 and G-200 groups, respectively ($P > 0.05$). Viable embryo numbers and ova/embryo recovery rate were greater ($P < 0.05$) in ewes of the G-200 (6.9 ± 1.1 and 67.8 %) than G-100 (1.0 ± 0.5 and 27.6 %) group. A dose of 200 mg pFSH was more effective in inducing a superovulatory response and embryo yield after NSER in ewes, however, the 100 mg dose was insufficient for these purposes.

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1. Introduction

Multiple ovulation and embryo transfer (MOET) programs have been conducted as part of the Lacaune genetic improvement program in France (Torres et al., 1987; Baril et al., 1995; Cognie, 1999; Baril et al., 2001) with the aim being acceleration of the multiplication of superior genotypes of this breed. There, however, are still some limitations to the large-scale use of the MOET techniques, the main ones being the large variability in the superovulatory response (Bartlewski et al., 2016), adhesions formed after surgical recovery of embryos (Forcada et al., 2011), and to the relatively greater cost of the technique when compared to the value of animals.

The ultimate variable for measuring the success of a superovulatory response is the number of viable embryos recovered. There have been developed effective cervical dilation procedures allowing transcervical uterine access and embryo recovery in ewes (Fonseca et al., 2019a). Results from repeated recoveries in which this technique was used indicated there was a similar number of viable embryos (Oliveira et al., 2020) compared to use of laparotomy for embryo recovery (Maciel et al., 2019; Pinto et al., 2020). These studies were conducted with Santa Ines ewes with 200 mg pFSH being administered to induce superovulation. Results were similar when these two procedures were used in a study in which there was comparison of the use of nonsurgical (transcervical) or surgical (laparotomy) embryo recovery methods in Santa Ines ewes (Santos et al., 2020). These results indicate that the non-surgical embryo recovery technique is reliable for accessing superovulation responses and viable embryo yield in sheep.

Superovulatory response can be affected by intrinsic factors such as breed (Torres et al., 1987; González-Bulnes et al., 2004), which indicates the importance for breed-specific treatments (Oliveira et al., 2012). Extrinsic factors also affect superovulatory response (Menchaca et al., 2009; Bartlewski et al., 2016), and some of these can be easily controlled, such as gonadotropin preparations, dosage regimen, and doses. Currently, FSH, obtained from porcine (pFSH) or ovine (oFSH) pituitary extracts is the primary choice for hormonal ovarian superstimulation in ewes (Bartlewski et al., 2016). Doses of 150–300 mg FSH are commonly used alone (Simonetti et al., 2008; Bartlewski et al., 2009) or in combination with eCG (Simonetti et al., 2008; Salehi et al., 2010; Oliveira et al., 2012, 2014). Doses smaller than 150 mg FSH were evaluated in Merino (Gibbons et al., 2010), Dorper (Loiola Filho et al., 2015), and Santa Inês (Menezes et al., 2014; Maciel et al., 2019; Rodriguez et al., 2019) breeds, resulting in overall similar ova/embryo recovery rates and number of viable embryos, compared to the use of larger doses.

After studying preovulatory follicular dynamics and ovulatory response, as well as embryo viability after non-surgical embryo recovery (NSER) in Lacaune ewes in which superovulation regimens were not imposed (Figueira et al., 2020a), strategic timepoints were identified for starting FSH administration, when there was either a 6 or 9-d progestogen treatment regimen, using a intermediary FSH dose (133 mg) (Figueira et al., 2020b). In this previous study, there was a larger number of viable embryos recovered after NSER when there was imposing of the 9- as compared with 6-d (3.9 and 1.8 viable embryos, respectively) treatment regimen. Furthermore, the imposing of the 9-d progestogen treatment regimen in Lacaune ewes did not result in adverse effects that would affect viable embryo production, such as anovulatory follicles, luteinized follicles or premature luteal regression, as reported for Santa Inês sheep (Rodriguez et al., 2019). In Santa Inês ewes, the administration of the 133 mg pFSH dose resulted in collection of fewer viable embryos (1.5), in comparison to when there was 200 (3.9) and 100 (2.6) mg of pFSH administred (Maciel et al., 2019). An adjustment of gonadotropin doses, using a 9-d progestogen/progesterone protocol for Lacaune ewes, therefore, is worthy ofinvestigation. In the present study, it was hypothesized that with a smaller dose of exogenous pFSH (100 mg) administration to lactating Lacaune ewes in which there was a 9-d progesterone treatment regimen along with NSER imposed, there would be satisfactory follicular recruitment, superovulatory responses, ova/embryo recovery, and production of viable embryos equivalent to what occurs with the traditionally used dose of 200 mg.

2. Materials and methods

2.1. Ethics and animal care

The Animal Care Committee of Embrapa Dairy Cattle approved the study design (protocol number # 2512100516/2016), and it was conducted in ways consistent with the principles of the Brazilian Society of Laboratory Animal Science.

2.2. Experimental conditions

This study was conducted during the breeding season, from April (Fall) to June (early Winter), on a commercial farm located at Soledade de Minas, (22°3′S, 45°2′W, and 938 m altitude), in Minas Gerais State, Brazil. According to Köppen-Geiger classification, the local climate is Cwa (Monsoon-influenced humid subtropical) with yearly temperatures ranging from 7 to 30 °C and average annual rainfall of 1600 mm.

The animals were maintained in pens and fed with corn silage (34.3 % of dry matter – DM, 6.5 % crude protein (CP), 44.7 % neutral detergent fiber (NDF), 4.2 % ash, 3.3 % fat. A nutrient-balanced concentrated supplement (800 g, with 18 % of CP) was provided twice a day to complete their nutritional (maintenance and lactation) requirements (National Research Council, 2007). Mineralized salt (DeHeus®, Rio Claro, Brazil), and drinking water was available *ad libitum*.

2.3. Experimental design

This study was conducted in a crossover experimental design with there being 60-d between the time periods of imposing the initial

and subsequent treatment regimens for induction of superovulation. The first embryo recovery session occurring on April, and the second session occurring in June (two replicates). Lactating Lacaune ewes ($n = 24$) of which two were primiparous and 22 were multiparous were studied. At the beginning of experiment, ewes were 129.2 ± 7.1 d into the lactational period and were producing 1.2 ± 1.0 kg milk/day. Ewe average body weight (BW) was 66.4 ± 1.3 kg and body condition score (BCS, 1–5 scale, 1 = emaciated, 5 = obese, Suiter, 1994) was 3.5 ± 0.04 (mean ± SEM).

In the first replicate, ewes were randomly allocated into two treatment groups in which there was super stimulation of follicular development with treatments consisting of either a total of 100 mg (G-100) or 200 mg (G-200) pFSH (Folltropin®-V; Bioniche Animal Health, Belleville, Canada). After 60 d from the time of the initiation of treatments of ewes of the first replicate, ewes there was imposing of the same treatment regimen for estrous synchronization, superovulation and embryo recovery, however, treatments of each ewe were the opposite of what these had been in the first replicate.

For estrous synchronization, an intravaginal silicone device containing 0.36 g progesterone (Primer PR®, Tecnopec, São Paulo, Brazil) was inserted (D0) and maintained for 9 d, and 24 h before removal, 37.5 µg laterovulvar (l.v.) D-cloprostenol was administered. Treatments to induce superovulation started 60 h before progesterone device removal (D6.5) and was administered twice a day (08:00 and 20:00), in six doses with there being lesser dosage amounts (25 %, 25 %, 15 %, 15 %, 10 % and 10 % of total dose) as the period of pFSH administration advanced. On D10 (24 h after device removal), 50 µg i.m. gonadorelin (Gestran®, Tecnopec, São Paulo, Brazil) were administrated. Estrous behavior was evaluated twice a day (08:00 and 20:00), for as long as 96 h after progesterone device removal, using fertile rams (1:4 ram-ewe ratio). Estrous onset was defined as the time when an ewe first stood to be mounted by a ram. Mating was repeated every 12 h until ewes would no longer stand to be mounted. There is a simplified schematic depiction of experimental procedures in Fig. 1.

2.4. Ovarian ultrasonography

Transrectal ovarian ultrasonography was conducted using an 8.0 MHz linear probe (M5VET, Mindray®, Shenzen, China) to assess number and diameter of detectable antral follicles and number of corpora lutea (CL) on the day of the first (D6.5 after device insertion) and second pFSH administrations (D7). Ovarian follicles were measured using electronic calipers, and the diameter was defined as the average of length and width of each follicle. The number of follicles were classified according to four categories (≤3.0 mm, 3.1–4.0 mm, 4.1–5.0 mm, and >5.0 mm). On D15 (6 days after device removal) an ultrasonography evaluation was performed using color Doppler mode for ascertaining which were luteinized structures, into categories of CL and luteinized anovulatory follicles (LAF). The LAF were defined as ovarian structures greater than 5.0 mm with a luteinized wall and cavity size that was larger than 50 % of the diameter of the structure. This approach is based on studies of Bartlewski et al. (2017) in which an LAF was considered to be an ovarian structure of ≥5 mm and lacking an ovulatory stigmata, and of a study of Oliveira et al. (2018) in which B and Color Doppler mode ultrasonographic techniques were used to quantify the LAF in ewes after imposing a superovulation treatment regimen. The proportion of LAF was calculated by dividing the number of LAF by number of luteinized structures (Maciel et al., 2019). This assessment allows for determination of the location of the first uterine flushing ipsilateral to the ovary with larger CL count.

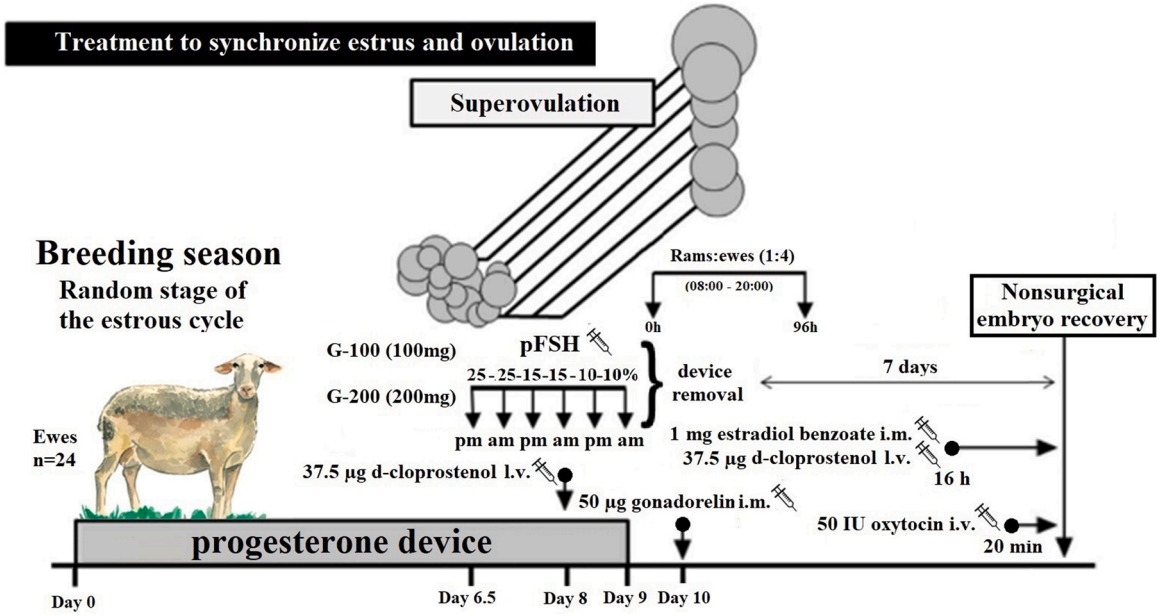

Fig. 1. Schematic representation of the procedures used in a crossover experimental design (two replicates 60-d apart) to assess either 100 (G-100) and 200 (G-200) mg of pFSH for superovulation in Lacaune ewes, submitted to non-surgical embryo recovery (NSER, after cervical dilation treatment*); i.m. intramuscular, i.v. intravenous, l.v. laterovulvar.

2.5. Embryo recovery and morphological evaluation

Embryo recovery was performed on D16 (7 days after device removal) in ewes having three or more CL (G-100, $n = 11$ and G-200, $n = 22$) utilizing the procedure for cervical dilation previously utilized in Lacaune ewes (Figueira et al., 2020a). The duration of time needed for transcervical penetration was considered to be the time needed for passage through the cervical rings of a Hegar dilator and catheter. The time needed for uterine flushing was considered to be the time required for flushing the initial uterine horn in which flushing occurred added to the time for the second cervical penetration with catheter and flushing of the second uterine horn. These durations in time were recorded and summed to determine the total time of the embryo recovery procedure (mean ± SEM). These times were considered to represent the efficiency determinations for NSER. All the ova/embryo recovered (unfertilized oocytes, embryos of different developmental stages, and zona pellucida) were listed, and embryos were transferred to a maintenance medium (Holding Plus®, Cultilab, Campinas, Brazil). Embryo evaluations were performed using a stereomicroscope with magnification of X 40 (Nova®, model XTD-20, Piracicaba, Brazil) while using the same criteria as those for cattle (Stringfellow and Givens, 2010). Grade 1, 2, and 3 embryos were considered to be viable embryos. Grade 1 and 2 embryos were considered to be those that were eligible for cryopreservation and were cryopreserved using either vitrification (Gibbons et al., 2011) or slow freezing (Fonseca et al., 2018) techniques for the study of post-transfer viability (Figueira et al., 2019). The following rates were calculated to assess the efficacy of the NSER: transcervical penetration rate = number of ewes that had the cervix completely penetrated for uterine flushing/number of ewes in which cervical penetration was attempted x 100; successful recovery rate = number of ewes having at least one ovum or embryo recovered/number of ewes that had the cervix completely penetrated for uterine flushing × 100; ova and embryo recovery rate = total number of recovered ova and embryo/number of CL × 100; and viable embryo rate = number of viable embryos/total number of recovered ova and embryo × 100.

2.6. Statistical analyses

Data were analyzed using SAS software (Statistical Analysis System®, version 9.3, SAS Inst., Inc., Cary, NC, USA). The statistical models to study the responses occurring during the crossover experimental design were similar to that proposed by Kaps and Lamberson (2017). The models included order (sequence of applied treatments), treatment and replicate as fixed effects, and ewe nested in order, as random effect.

Quantitative data as estrous length, interval from device removal to estrus, time of cervical penetration, and total time for conducting the procedure were analyzed using generalized linear models, with Gamma distribution and log link function using the GLIMMIX procedure. The numbers of CL, LAF, total follicles, and follicles based on diameter categories (≤3.0 mm, 3.1–4.0 mm, 4.1–5.0 mm, >5.0 mm), recovered ova/embryo, and viable embryos were also analyzed using the GLIMMIX procedure, however, considering the Poisson distribution and log link function.

Qualitative data from ewes with follicles >5.0 mm and ewes with a CL at the beginning of pFSH treatment, and viable embryos rate were analyzed using the GLIMMIX procedure, with binomial distribution and logit link function. The variables donors in estrus, percentage of responding donors (≥3 CL), transcervical penetration rate, successful recovery rate, and ova/embryo recovery rate were analyzed using the Chi-square test. Results are reported as means ± standard error of the means (MEANS ± SEM). There were considered to mean differences when there was a $P < 0.05$.

3. Results

There was no treatment effect ($P > 0.05$) on the percentage of ewes with CL or follicles >5.0 mm at the beginning of the pFSH administration (Table 1). The total number of follicles and follicular count in each diameter category (≤3.0 mm, 3.1–4.0 mm, 4.1–5.0 mm, >5.0 mm) did not differ ($P > 0.05$) between treatment groups at the time of the first pFSH administration (Fig. 2). There was a larger ($P < 0.05$) number of 3.1–4.0-mm follicles in the ewes of the G-200 (3.9 ± 0.5) than G-100 (2.7 ± 0.4) treatment group at

Table 1
Values for ovarian response variables (MEANS ± SEM) at the first and second injections of 100 (G-100) or 200 (G-200) mg of pFSH* (six decreasing doses, starting 60 h before device removal) during progesterone-based treatment regimen for 9 d in Lacaune ewes, in a crossover-designed experiment of two replicates 60-d apart.

Variables	Groups G-100 ($n = 24$)	G-200 ($n = 24$)	*P*-value
First pFSH injection			
Total number of antral follicles	10.9 ± 0.9	11.2 ± 0.9	0.80
Ewes with follicles > 5.0 mm (%)	62.5 ± 10.1	45.8 ± 10.4	0.23
Ewes with corpora lutea (%)	37.5 ± 10.1	66.7 ± 9.8	0.06
Second pFSH injection			
Total number of antral follicles	11.8 ± 1.0	13.5 ± 0.9	0.10
Ewes with follicles > 5.0 mm (%)	62.5 ± 10.1	62.5 ± 10.1	0.96
Ewes with corpora lutea (%)	37.5 ± 10.1	58.3 ± 10.3	0.16

*Administered in six decreasing doses (25, 25, 15, 15, 10 e 10 %) starting at 60 h (first dose) and 48 h (second dose) before device removal.
[ab]Denotes significant differences between treatments ($P < 0.05$).

the time of the second pFSH administration, but there were no differences ($P > 0.05$) between treatment groups for the other follicular diameter categories.

The percentage of ewes in estrus was greater ($P < 0.05$) in the G-200 compared to G-100 treatment group (Table 2). There was a treatment effect ($P < 0.05$) for estrous length, with greater values in ewes of the G-200 group. There was a greater responsiveness ($\geq$3 CL) to the superovulatory treatment regimen in ewes that were administered a 200 mg FSH dose. There was, therefore, a larger ($P < 0.05$) number of luteinized structures (CL plus LAF) in ewes of the G-200 compared to G-100 group. The proportion of LAF was greater ($P < 0.05$) in the ewes of the G-100 compared to G-200 treatment group.

Transcervical penetration and uterine flushing were successfully conducted in 94 % (31/33) of the ewes and this rate did not differ ($P < 0.05$) between treatment groups (Table 2). Although there were no differences ($P > 0.05$) in number of cervical rings between ewes of the G-100 and G-200 groups, the time required for transcervical penetration was longer in the ewes of the G-100 than G-200 group. The total time required for performing the NSER procedure was similar ($P > 0.05$) for ewes in both treatment groups. Both proportion of ova/embryo recovery and successful embryo recovery rate were less ($P < 0.05$) in ewes of the G-100 than G200 group. The numbers of recovered ova/embryo, morulae, blastocysts, unfertilized ova, viable embryos, and freezable embryos were larger ($P < 0.05$) for ewes of the G-200 than G-100 groups.

4. Discussion

The pattern of ovarian follicular development (number and diameter of visible follicles) and CL count were similar at the time of first FSH administration in ewes of both treatment groups. The quantity of the FSH dose administered during the superovulation treatment regimen, therefore, is the likely reason for the larger number of 3.1–4.0-mm follicles in ewes of the G-200 group that was observed 12 h after the first pFSH administration. The smaller FSH dose has been associated with a lesser follicular recruitment leading to a lesser ovulation rate and consequently number of viable embryos (Boscos et al., 1997). At the initial time of pFSH treatment, the relatively smaller dose of this gonadotropin was probably not sufficient to effectively induce follicle recruitment, therefore, this the likely explanation for the superovulation outcome when there was the G-100 treatment. In a previous study, there was a positive correlation between number of medium-sized (4.0 mm in diameter) antral follicles 12 h after the first pFSH administration, and number of viable recovered embryos (Bartlewski et al., 2008). The 50-mg pFSH dose (25 % of the entire dose) at the time of the first administration is the likely cause for the larger number $\cong$ 4 mm diameter follicles, which likely contributed to the larger number of viable embryos recovered in ewes of the G-200, compared with the G-100 group.

Estrous length was longer in the ewes of the G-200, compared with the G-100 treatment group. Because the largest dose of pFSH

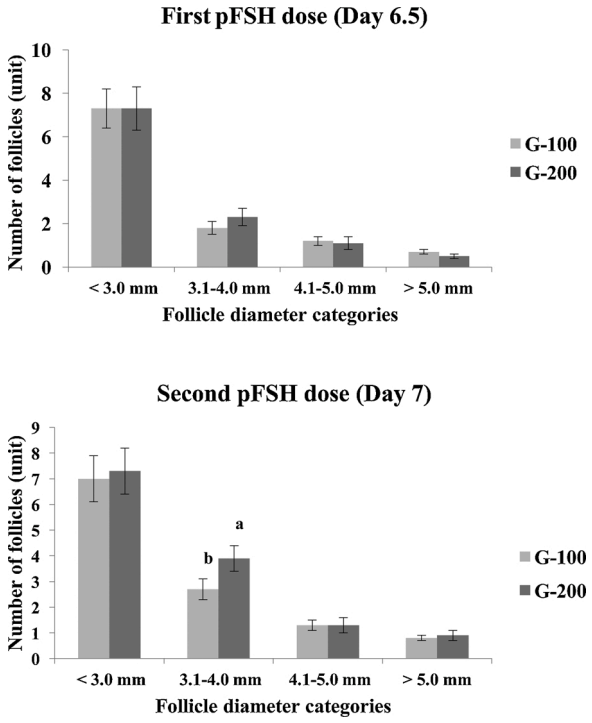

Fig. 2. Number of follicles (MEANS ± SEM) grouped by diameter ($\leq$3.0 mm, 3.1–4.0 mm, 4.1–5.0 mm, >5.0 mm) at the time of the first (Day 6.5) and second injections (Day 7) of pFSH, during a progesterone-based treatment regimen for 9 d to estrous synchronization in Lacaune ewes; Total pFSH: 100 mg (G-100, light gray) or 200 mg (G-200, dark gray) proportioned into six doses (25 %, 25 %, 15 %, 15 %, 10 % and 10 % of total FSH) administered every 12 h; [ab] Denotes differences between treatments ($P < 0.05$).

Table 2
Estrous behavior expression, superovulatory response and embryo recovery variables in Lacaune ewes treated with 100 (G-100) or 200 (G-200) mg pFSH* during a progesterone-based treatment regimen for 9 d in Lacaune ewes, in a crossover-design experiment of two replicates 60-d apart.

Variables	Groups G-100 ($n = 24$)	G-200 ($n = 24$)	*P*-value
Percentage of ewes in estrus (%)	66.7 (16/24)	100.0 (24/24)	<0.01
% of responding donors (with ≥ 3 CL)	45.8 (11/24)	91.7 (22/24)	0.02
Interval from device removal to estrus (h)	31.5 ± 1.9 (16)	26.5 ± 1.6 (24)	0.07
Estrus length (h)	24.0 ± 3.4 (16)	32.0 ± 2.5 (24)	0.03
Luteinized structures (n)	4.2 ± 0.8 (24)	13.8 ± 1.3 (24)	<0.01
Corpora lutea (n)	2.6 ± 0.7 (24)	11.6 ± 1.2 (24)	<0.01
Luteinized anovulatory follicles (n)	1.4 ± 0.3 (24)	2.2 ± 0.6 (24)	0.04
% of luteinized anovulatory follicles	34.0 (34/100)	16.0 (53/331)	<0.01
Transcervical penetration (%)	100 (11/11)	90.9 (20/22)	0.54
Cervical rings (n)	7.1 ± 0.3(11)	7.6 ± 0.3 (20)	0.83
Time of transcervical penetration (min)	7.0 ± 2.0 (11)	3.9 ± 0.4 (20)	<0.01
Total time of procedure (min)	31.0 ± 2.2 (11)	26.3 ± 1.0 (20)	0.14
Corpora lutea in ewes collected (n)	4.6 ± 1.1 (11)	12.8 ± 1.1 (20)	<0.01
Successful recovery[c](%)	63.6 (7/11)	100.0 (20/20)	0.01
Number of recovered ova/embryo	1.3 ± 0.5 (11)	8.7 ± 1.1 (20)	<0.01
Ova/embryo recovery[d](%)	27.6 (14/51)	67.8 (173/255)	<0.01
Unfertilized ova (n)	0.2 ± 0.2 (11)	1.7 ± 0.8 (20)	<0.01
Morulae (n)	0.5 ± 0.4 (11)	4.0 ± 1.1 (20)	<0.01
Blastocysts (n)	0.5 ± 0.2 (11)	3.1 ± 0.8 (20)	<0.01
Viable embryos (n)	1.0 ± 0.5 (11)	6.9 ± 1.1 (20)	<0.01
Freezable embryos (n)	1.0 ± 0.5 (11)	6.5 ± 1.0 (20)	<0.01
Viable embryos[e](%)	78.5 (11/14)	79.8 (138/173)	0.97

*Step-down – six decreasing doses (25, 25, 15, 15, 10 and 10 %), starting 60 h before device removal).
[ab]Denotes significant differences between treatments ($P < 0.05$).
() Number of animals.
[c] Successful recovery rate: (number of ewes having at least one ovum or embryo recovered/number of ewes that had the cervix completely penetrated for uterine flushing) × 100.
[d] Ova/embryo recovery rate: (Total number of recovered ova and embryo/total number of corpora lutea) × 100.
[e] Viable embryos rate: (Number of embryos Grade 1, 2 or 3 per ewe/number of recovered ova/embryo per ewe) × 100.

resulted in greater follicular recruitment, it is likely that secretion of 17β-estradiol, which is a primary factor in inducing estrous behavior, was also greater in the ewes of G-200 treatment group. In a previous study, there was a positive correlation between serum 17β-estradiol concentrations at the time of the second pFSH administration and the number of luteal structures before collection (Bartlewski et al., 2008). In Dorper ewes, there were differences in values related to estrous variables following treatments to induce superovulation with either 128 or 200 mg of pFSH (Loiola Filho et al., 2015). In this previous study, ewes treated with the larger pFSH dose (200 mg) expressed estrus in a shorter period of time after initiation of the pFSH treatments than ewes treated with a smaller dose of pFSH (128 mg). Furthermore, the larger number of LAF could also be associated with the longer length of estrus observed in the current study, because ovarian structures from which there is not ovulation can secrete estrogens (Okada et al., 2000; Veiga-Lopez et al., 2006).

Although there was a larger absolute number of LAF in the ewes of the G-200 group, there was a larger proportion of LAF (per total luteinized structures) in the ewes of the G-100 treatment group. This indicates that the smaller dose of pFSH was insufficient to induce recruitment of a large number of follicles and did not induce adequate follicular development and ovulation. Not only FSH dose, but other factors, such as variability in the release pattern of LH secretion in ewes treated to induce superovulation, may be related to the occurrence of LAF (Rodriguez et al., 2019). In Santa Inês ewes, LH supplementation at the end of treatment regimen for superovulation led to an increased proportion of ewes with more larger numbers of ovulations (11 or more CL) and a lesser proportion of LAF relative to total luteal structures (Oliveira et al., 2012).There was, however, no effect on the ovulation rate in ewes of the Manchega, Churra and Merino breeds treated with LH at the end of the superovulatory treatment regimen (Picazo et al., 1996). The results from these studies indicate that the FSH/LH ratio at the end of superovulatory treatment regimen may result in inconsistent responses, and that ovulation rate and embryo production variations appear to be related to intrinsic variability of ewes of different breeds (Oliveira et al., 2012). When there were the experimental conditions of the present study, administration of a GnRH analogue to induce ovulation appeared to have a favorable effect in Lacaune ewes treated with 200 mg pFSH, because even though there was a larger number of LAF and longer length of estrus, ewes had a lesser proportion of LAF.

The treatment that induced the larger superovulatory response (G-200) in the present study also allowed for a more time-efficient cervix penetration, and therefore, there was the shortest time needed for conducting these procedures. The cervix contains FSH receptors and it is suggested that LH and FSH functionally modulate the cervical physiology during estrus, by increasing local concentrations of cyclooxygenase 2 (COX2; Leethongdee et al., 2016). The enzyme COX2 is part of the pathway for PGE_2 synthesis, which is probably the central pathway for cervical relaxation during estrus (Falchi and Scaramuzzi, 2015). It, however, seems unlikely that LH and FSH induce complete cervical relaxation, nonetheless these gonadotropins might function secondarily to the actions of oxytocin and estradiol (Leethongdee et al., 2016). Because with both treatments in the present study, there was the same hormonal

cervical relaxation treatment regimen (with exogenous D-cloprostenol, estradiol benzoate and oxytocin), endogenous hormones might have had major effects in facilitating cervical penetration. Dias et al. (2020) observed differences in time required for cervical penetration using a similar dose of oxytocin but varying estradiol doses, and therefore, provided evidence that the hormonal milieu is important for efficiently conducting NSERs.

The greater response of ewes to the superovulation treatment regimen in the G-200 treatment group led to recovery of a larger number of viable and/or freezable morulae or early blastocysts. This is the most relevant outcome of the *in vivo* embryo production technique. The number of unfertilized ova was also greater in the ewes of the G-200 that G100 treatment group. Loiola Filho et al., 2015 also reported that there was an increase in unfertilized ova with the largest dose of FSH. Unlike results in this previous study, there were no indications that the larger number of unfertilized ova in the current study was associated with a lesser total yield of viable embryos that were of a quality worthy for freezing. Because several intrinsic and extrinsic factors affect the superovulatory response (Bartlewski et al., 2016), comparing results from different studies has limited value. It, however, is likely that the use of the GnRH analogue in the current study had beneficial effects as a result of a reduction in number of unfertilized ova which is likely associated with the increasing embryo yield, as reported previously by Menchaca et al. (2009).

The proportional rate of ova/embryo recovery in ewes of the G-200 treatment group was larger than in ewes of the G-100 group (67.8 % compared with 27.6 %) and was similar to the percentage (65 %) reported in other studies where NSER was conducted in ewes after imposing superovulation treatment regimens (Barry et al., 1990; Mylne et al., 1992). The greater the superovulatory response, the greater the probability of recovery of least one ovum or embryo. In the current study, 100 % of the ewes in the G-200 group that had cervix penetration had at least one ovum or embryo recovered. In Lacaune ewes where there were not treatments to induce superovulation, this rate ranged from 46 % to 89 % (Figueira et al., 2020a). Results from the present study, therefore, are consistent with the superovulatory response and adequate luteal function that has occurred when conducting previous studies where there was effective and efficient NSER after imposing cervical dilation treatment regimens (Fonseca et al., 2019b; Figueira et al., 2020a). Further studies are needed to determine the extent in which the recovery efficiency using the NSER technique is affected by the number of ovulations and/or by hormonal profile, in different ewe breeds.

The ova/embryo recovery rate in ewes of the G-200 treatment group (68 %) was slightly less than rates reported when there was laparotomy recovery following superovulation using a similar, as compared to that of the present study, FSH treatment regimen in ewes of the Lacaune (77 %; Torres et al., 1987) and Santa Ines (77 %; Oliveira et al., 2014) breeds, however, similar to the rates in ewes of the Corriedale (Simonetti et al., 2008) and Dorper (Loiola Filho et al., 2015) breeds. The average 6.9 viable embryos collected in the present study when there was treatment with 200 mg pFSH and with use of NSER procedures approximates to the world average of 6.3 embryos per flushing procedure (International Embryo Transfer Society, 2019). Furthermore, this value was similar to that previously reported for Lacaune ewes on which there was imposed a long-term progestogen-based treatment regimen (13.5 d) and superovulatory treatment with 16 mg of Armour pFSH equivalent and 500 IU eCG (Torres et al., 1987).

The possibility of performing successive embryo collections in a short period of time, with the use of NSER, increases the potential for progeny production, when compared to surgical collections, if NSER can be conducted in ways that there are not risks of compromising the subsequent fertility in ewes (Fonseca et al., 2019a). Quality of the embryos produced in the present study was evaluated after slow freezing or vitrification cryopreservation techniques, and reasonable survival and pregnancy rates after fixed-time transfer could be achieved using the slow freezing regimen (Figueira et al., 2019).

Collectively, the results from previous and present studies when there was NSER in Lacaune ewes indicates adjusting length of the progestogen usage to 9d (Figueira et al., 2020a, b) and customizing the pFSH doses leads to improvements in ova/embryo recovery rates and embryo yield. When all results are considered, NSER-based MOET programs and embryo cryopreservation are feasible and can lead to successful outcomes in sheep production. When there was manufacturing readiness level/technology readiness level (TRL/MRL) assessments (Mankins, 1995), there is the inference that sequential studies on MOET utilization in sheep flocks supported the technology development workflow to move from adaptation phases (Scale 4 and 5) to conclusion of the validation phase (Scale 6). The next actions include refinements and commercial use of the product.

5. Conclusions

The dose of 100 mg pFSH was insufficient for desirable superovulation responses in Lacaune ewes, while administration of 200 mg pFSH resulted in an optimal superovulatory response and embryo yield after NSER. Furthermore, there were only a desirable ova/embryo recovery rate when there were greater superovulatory responses. The use of 200 mg pFSH for superovulation, associated with NSER, is an effective strategy for increasing offspring yield from Lacaune ewes of high genetic merit when conducting MOET programs.

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1 that there has been no duplicate publication or submission elsewhere of this work
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Declaration of Competing Interest

The authors reported no declarations of interest.

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