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Preovulatory follicular dynamics, ovulatory response and embryo yield in Lacaune ewes subjected to synchronous estrus induction protocols and non-surgical embryo recovery

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ABSTRACT

The objective of this study was to assess the effect of the duration of progesterone-based estrus induction protocols on preovulatory follicular dynamics, ovulatory response, and embryo yield after non-surgical embryo recovery (NSER) in Lacaune ewes. Females received acetate medroxyprogesterone intravaginal sponges for six (G-6; n = 14) or nine (G-9; n = 14) days plus d-cloprostenol and eCG 24 h before sponge removal (Day 0). Preovulatory follicular dynamics and the luteal characteristics are evaluated by B-mode and Color-Doppler ultrasonography. NSER was performed five to six days after ovulation. The estrous behavior rate was 85.7% for both groups, and the percentage of ewes that ovulated was 92.9% in G-6 and 100% in G-9. The day of wave emergence (relative to Day 0) did not differ ($P > 0.05$) between G-6 (-3.0 ± 0.5) and G-9 (-4.2 ± 0.5). The number of follicles of size 4.1–5.0 mm was higher ($P < 0.05$) in G-9 (1.4 ± 0.2) compared to G-6 (0.8 ± 0.2) during the Days −4 to 0. At NSER, the transcervical penetration rate was 95.2% (20/21) and its duration time was lower ($P < 0.05$) in G-9 (3.4 ± 0.6 min) than in G-6 (7.2 ± 1.3 min). The number of ovulations and viable embryos was higher ($P < 0.05$) in G-9 (2.9 ± 0.3 and 1.3 ± 0.4, respectively) than in G-6 (1.9 ± 0.3 and 0.4 ± 0.2, respectively). In conclusion, the 9-day protocol promoted higher ovulation rate and embryo yield; moreover, the cervical dilation treatment allowed NSER in a high percentage of Lacaune ewes.

1. Introduction

Lacaune is one of the main breeds of sheep raised for dairy farming, having become favored worldwide due to the genetic gains achieved by the French genetic improvement program [1]. In order to accelerate herd multiplication and genetic improvement, it is essential to use certain reproductive biotechnologies, namely estrus induction [2,3], artificial insemination [1] and multiple ovulation and embryo transfer (MOET) [4,5].

Due to the sheep's seasonal status [6], estrus induction protocols are required in the non-breeding season [7]. These treatments, when applied over a long period (12–14 days), result in satisfactory estrus induction, but variable fertility [8,9]. Short-term (5–7 days) protocols are an alternative that offer similar or better fertility rates [10], with benefits including lower ovulation of persistent dominant follicles [11], minor hormonal residual effects [12], a lower likelihood of losing the intravaginal sponge/device [13], as well as the option of inducing the ovulation of new follicles from the first

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follicular wave [14], given that there are a distinctive pattern of follicular wave dynamics during the treatment period [15]. Few studies exist focusing on the progesterone-based estrus induction protocols of different durations in dairy ewes. In the Awassi breed, ewes treated with progesterone (P_4) devices for 6, 9 or 12 days showed similar pregnancy and lambing rates [16], while in dairy Lacaune ewes there was a tendency to achieve the highest lambing rate after a long-term (12 days) compared to a short-term (6 days) progesterone treatment [2]. A fine adjustment of progesterone/progestogen exposure time in dairy Lacaune ewes may improve reproductive results, by the stimulation of different ovulatory follicular wave grown under different hormonal conditions, which also provides better support to other reproductive biotechnologies. However, there is still no in-depth information available to support this summation.

Laparotomy is the worldwide technique of choice for embryo recovery in ewes [17]. This method demands prior fasting and anesthetic drugs that subject the animal to certain risks. Furthermore, adhesions, post-operative trauma, and stress can arise from the procedure, limiting successive recoveries. Therefore, efforts are being made to enable MOET programs based on non-surgical embryo recovery (NSER) by cervical route, due to growing concerns about animal welfare and to improve the applicability of these biotechnologies [18,19]. In Brazil, studies with Santa Inês ewes reached lower transcervical penetration rates of 61% [20] and 81% [19], compared to 95% in Dorper ewes [21]. However, considering the difference in transcervical penetration rates among breeds [22], the efficiency and repeatability of the NSER need to be carefully evaluated when applied to different breeds [18]. The NSER has not yet been reported for Lacaune ewes.

The main objective of this study was to evaluate the effects of the duration of progesterone-based estrus induction protocols (6 or 9days) on preovulatory follicular dynamics, ovulatory response and embryo yield after non-surgical embryo recovery in Lacaune ewes.

2. Material and methods

2.1. Experimental conditions

This research was approved by the Animal Care Committee of Embrapa Dairy Cattle (protocol 2512100516/2016). The study was conducted during the period of lengthening daylengths [15] (September–October) in a commercial farm located in Soledade de Minas, Minas Gerais State, Brazil (latitude 22°3′ S, longitude 45° 2' W and at an altitude of 938 m). Dairy ewes of the Lacaune breed (n = 28) based in good sanitary and clinical conditions were used. The animals were kept in collective pens and were fed twice a day with a diet based on silage corn and balance concentrated to meet their nutritional requirements [23]. Mineralized salt (DeHeus®, Rio Claro, Brazil) and water were offered *ad libitum*. The ewes were 15–93 months old, had 67.2 ± 2.0 kg (mean ± SEM) of bodyweight, 3.4 ± 0.1 units of body condition score (BCS–scale from 1 to 5, where 1 = emaciated and 5 = obese) [24] and 89.1 ± 6.2 days in milk at the beginning of the experiment.

2.2. Induction of synchronous estrus, estrus detection, and mating

Ewes were randomly separated into two experimental groups according to their parity (14 primiparous and 14 multiparous) and bodyweight (G-6: 69.7 ± 2.9 and G-9: 63.9 ± 2.0 kg). Synchronized estrus induction treatment consisted of intravaginal sponges containing 60 mg of medroxyprogesterone acetate (MAP, Progespon®, Zoetis, Campinas, Brazil) for six (G-6; n = 14) or nine (G-9; n = 14) days. The MAP sponges were inserted on a random day of the estrous cycle or anovulatory period. At 24 h before sponge removal, in both treatments, 400 IU of eCG i.m. (Folligon 5000IU®, Intervet, São Paulo, Brazil) and 37.5 μg of d-cloprostenol (a synthetic analog of $PGF_{2\alpha}$, Prolise®, Tecnopec, São Paulo, Brazil) latero-vulvar (l.v.) were administered. The sponge removal day was considered as Day 0 to support data analyses.

After sponge removal, estrous behavior was recorded by using healthy and fertile rams (ratio of 1 ram: 6 ewes) twice a day (08:00 and 18:00). The rams were rotated in both treatments to minimize male fertility variations. Ewes remained with the ram at least 30 min in each observation period if no mounting acceptance occurred. Estrus onset was defined as the time when the ewe first stood to be mounted by the ram. Mating was repeated every 12 h until no mounting acceptance.

2.3. Ultrasonographic evaluations

B-mode transrectal ovarian ultrasonography (M5VET, Mindray®, Shenzen, China, 8.0 MHz) was carried out daily during exogenous progestogen treatment to determine the presence of corpora lutea (CL) and the follicular population. On MAP sponge insert day, the Color-Doppler mode ultrasonography was performed in animals with CL to evaluate the luteal functionality [25]. In each exam, the number, position, and diameter of ovarian follicles (≥2 mm in diameter) were recorded on an individual ovarian map to follow the sequential follicular development. This data was used for retrospective evaluation of follicular dynamics. The day of emergence of an antral follicle was the day that the follicle was 2 mm in diameter, with an increase to ≥3 mm on the next day. The day of emergence of the largest ovulatory follicle was considered as the day of emergence of the ovulatory follicular wave. After sponge removal, evaluations were performed twice a day to measure the ovarian antral follicles and to track the moment of ovulation, which was considered as the average period between the last exam at which the first preovulatory follicle was observed and the first exam at which it was no longer seen. The growth rate was calculated for the two largest ovulatory follicles, which was considered to be the difference between the maximum and minimum diameter of the ovulatory follicle divided by the duration of the growth phase. The duration of the follicular growth phase was the time taken (in days) to grow from 2 to 3 mm until reaching the ovulatory diameter.

Seven days after sponge removal, an ultrasound evaluation was performed using B-mode and Color-Doppler mode ultrasonography for counting and measuring the CL, and to calculate the total and vascularized luteal tissue areas. The area (cm^2) of each CL was measured using the ultrasound device calipers (ellipse and trace tools). The luteal area and the cavity were measured using the ellipse tool, and the luteal tissue area was obtained by subtracting the area of the cavity of the total luteal area. The vascularized luteal area was defined using the trace tool. Values were summed up in ewes with >1 CL for the analysis of total luteal tissue area and total vascularized luteal area. A percentage of vascularized luteal tissue area was also established (total vascularized luteal area [cm^2]/total luteal tissue area [cm^2] * 100). This assessment allowed for determining the direction of the first uterine flushing, ipsilateral to the ovary with a higher CL count or a greater vascularized percentage. Thirty days after embryo recovery, the genital tract was evaluated by ultrasonography again, to verify the health of the cervix, uterus, and ovaries (with presence of altered echotexture being suggestive of abnormality).

2.4. Cervical dilation and transposing

All naturally-mated ewes (n = 24) were submitted to the cervical dilation protocol 4–5 days after ovulation, containing: 1 mg of

estradiol benzoate (Sincrodiol®, Ouro Fino, Cravinhos, Brazil) i.m. and 37.5 μg of d-cloprostenol (a synthetic analog of PGF2α, Prolise®, Tecnopec, São Paulo, Brazil) l.v. were administered at 16 h before NSER. Twenty minutes before the cervical penetration attempt, 50 IU of oxytocin (Ocitocina forte®, UCB, São Paulo, Brazil) was also administered intravenously (i.v.) (Fig. 1). Immediately after oxytocin, 40 mg of hyoscine-N-butylbromide and 5 g of sodium dipyrone (Buscofin®, Agener Union, Embu-Guaçu, Brazil) were administered by both i.v. and i.m. routes, respectively. In addition, 1 mg/kg of acepromazine maleate (Acepran 1%®, Vetnil, Louveira, Brazil) i.m. was administered immediately after oxytocin. This sedation continued for 45 min; enough time for carrying out the procedure.

After sedation, the ewes were kept in a standing position and contained in an appropriate cart to avoid lateral and dorsal-ventral movements. Transcervical embryo recovery was performed by the same experienced technician, as described by Fonseca et al. [18]. The external *os* of each cervix was classified into one of six types, with few modifications as earlier described [26], based on surrounding folds of tissue. The classification was as follows: duckbill – two opposing folds, the flap – one-fold, rosette – cluster of folds, slit – no projection, papilla – small rounded protuberance, and mixed – no pattern or combination of two types.

During the cervical penetration attempt, the number and geographic-anatomical arrangement of rings were recorded to create a cervical map. The animals were then classified according to the degree of cervical misalignment (one –rectilinear cervix, two – intermediate, and three – highly asymmetrical) [27]. The time of transcervical penetration (considered to be the time to transpose the cervical rings with Hegar dilator and catheter) and uterine flushing (considered as the time for flushing the first uterine horn added to the time for the second transcervical penetration with catheter and flushing of the second uterine horn) were recorded and calculated to determine the total time of embryo recovery procedure. At the end of the procedure, the ratio of fluid recovered in a graduated cylinder and the fluid inputted by graduated syringe was calculated for an evaluation of the fluid recovery rate.

All the structures recovered (non-fertilized oocytes, embryos of different developmental stages, and zona pellucida) were listed and the embryos were transferred to maintenance (Holding Plus®, Cultilab, Campinas, Brazil). Embryo evaluation was performed using a stereomicroscope with magnification: ×40 (Nova®, model XTD-20, Piracicaba, Brazil), following the same principles used for cattle, being Grade I: excellent or good; Grade II: fair; Grade III: poor; and Grade IV: dead or degenerating [28]. Embryos of grades I, II and III were considered viable embryos.

2.5. Statistical analyses

Parametric data obtained included the day of emergence of the ovulatory antral follicular wave, the duration of the follicular growth phase, the ovulatory diameter and growth rate of the largest and the second-largest ovulatory antral follicle, the estrus duration, the interval from sponge removal to estrus onset, the interval from sponge removal to ovulation, the interval from estrus onset to ovulation, the number of ovulations, the number of CL, the total luteal tissue area, the total vascularized luteal area, the percentage of vascularized area, the time of transcervical penetration, the time of uterine flushing, and the total time of embryo recovery procedure. All of these were analyzed using generalized linear models, with Gamma distribution and log-link function using GLIMMIX procedure in SAS® (Statistical Analysis Software, Cary, NC, United States of America). The model included bodyweight as a covariate, and treatment, category (primiparous and pluriparous) and interaction as fixed effects. The number of rings, recovered structures, unfertilized oocytes, morulae, blastocysts, viable embryos, and the cervix misalignment score were also analyzed using GLIMMIX procedure but considering Poisson distribution and log-link function as well. The model included treatment and category as fixed effects.

Data from follicular populations was analyzed from Day −4 to Day 0 relative to sponge removal. The population of antral follicles was divided into four categories according to follicular diameters (≤3.0 mm, 3.1–4.0 mm, 4.1–5.0 mm, and >5.0 mm), then repeated measure analysis was performed using PROC GLIMMIX, with Poisson distribution and log-link function for. The autoregressive and unstructured matrixes were applied to model the residual covariance. The model included category, day, treatment, ewe (as a random variable) and interactions where necessary. Multiple comparisons were made using the Tukey test.

Percentage data obtained included multiple ovulations rate (% of ewes that ovulated ≥ 3 CL), successful recoveries rate (% of ewes in which at least one structure was recovered), viable embryos rate (obtained dividing number of viable embryos by total number of structures recovered), and unfertilized rate (obtained dividing number of unfertilized by total number of structures recovered), which were analyzed using GLIMMIX procedure, with binomial distribution and logit link function. The model included treatment and category as fixed effects.

Descriptive statistics of estrous behavior, ovulation, cervical

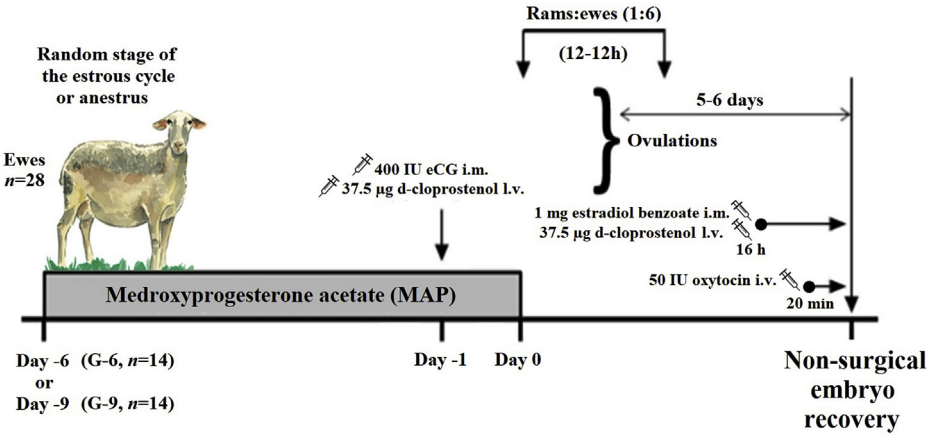

Fig. 1. Schematic representation of the experimental procedures used to assess two synchronous estrus induction protocols for six or nine days of progestogen exposure and to check the feasibility of non-surgical embryo recovery (NSER) in the Lacaune breed –eCG: equine chorionic gonadotropin; i.m: intramuscular; i.v: intravenous, l.v: laterovulvar.

retraction and transposition rates, ewes successfully flushed, fluid recovery rate, and embryo recovery rate (obtained by dividing the total number of recovered structures by the total number of corpora lutea) were presented. Results of generalized linear model analyses are shown as the least square means ± standard error of the means (LSMEANS ± SEM) and differences were considered significant from $P < 0.05$. Rates are expressed as percentages.

3. Results

3.1. Reproductive behavior and ovarian response

The percentage of ewes bearing CL at the beginning of the estrus induction protocols was the same (57.1% or 8/14) for both treatment groups. The emergence day of the ovulatory antral follicular wave relative to Day 0 (MAP sponge removal day) did not differ ($P > 0.05$) between G-6 (range −6 to −1) and G-9 (range −7 to −1), as shown in Table 1. The duration of progestogen treatment for six (G-6) or nine (G-9) days did not affect ($P > 0.05$) ovulatory follicular development (ovulatory diameters, durations of growth phase, and growth rates of the two largest follicles), or affect the intervals from sponge removal to estrus onset and to ovulation, or the estrus duration and interval from estrus onset to ovulation (Table 1). The number of ovulations and the percentage of multiple ovulations was higher ($P < 0.05$) in G-9 compared to G-6.

Antral follicular counts according to the diameter categories in the last days of MAP treatment in G-6 and G-9 groups are shown in Fig. 2. There was treatment × day interaction, with a decrease ($P < 0.05$) in follicle number ≤ 3.0 mm on Day 0 in relation to the previous day only in G-9. The number of follicles of 3.1–4.0 mm did not differ ($P > 0.05$) between treatments or days. On the other hand, there was a treatment effect ($P < 0.05$) on the follicle number with 4.1–5.0 mm, with a higher count in G-9 (1.4 ± 0.2) than in G-6 (0.8 ± 0.2). There was a day effect on the number of follicles >5 mm, which increased ($P < 0.05$) on Days −3 and 0 in both treatments compared to other days.

After MAP sponge removal, the percentages of estrous behavior in those ewes bearing CL and in those without CL at the beginning of the study were 87.5% and 83.3% in G-6, and they were 100% and 66.7% in G-9, respectively. Overall, the estrous behavior rate was 85.7% for both treatment groups. The percentage of ewes that ovulated was 92.9% in G-6 and 100.0% in G-9. Ovulations of follicles coming from the two last waves occur in 15.4% (2/13) of cases in G-6 and in 35.7% (5/14) in G-9. The interval between the emergence of these last waves was 4.0 ± 0.3 days. The percentage of estrus was lower than ovulation because three ewes (one from G-6 and two from G-9) had silent ovulation. These animals presented preovulatory follicles >5 mm at the time of eCG treatment and ovulated a few hours after sponge removal (24.0 ± 8.0 h). In G-6, one ewe had persistent dominant follicles and did not ovulate. The G-9 treatment induced a better ovulatory response, as measured by the number of ovulations and percentage of multiple ovulations (≥3 CL, Table 1), beside a higher ($P < 0.05$) total luteal tissue area in relation to G-6 (Table 2). The vascularized luteal area and percentage of vascularized luteal tissue area did not differ between groups.

3.2. Transcervical penetration and embryo recovery

Embryo recovery was conducted 5–6 days after ovulation (5.4 ± 0.4 days), though only in the 24 ewes that showed signs of estrus. The percentage of ewes for each type of cervical *os* was: duckbill (26%), rose (26%), flap (21%), slit (4%), papilla (9%), and mixed (9%). The external cervical *os* was not adequately visualized for clamping and retraction in 12.5% (3/24) of ewes. Cervical retraction rates reached 91.6% (11/12) in G-6 and 83.3 (10/12) in G-9. A cervical penetration attempt was performed in those ewes that had the cervix retracted and the transposition rate achieved was 100.0 (11/11) in G-6 and 90.0 (9/10) in G-9. In the only ewe that transcervical penetration was not possible with the catheter, the dilator Hegar was successfully transposed. In three animals of the G-6 treatment group, the cervix was penetrated for uterine flushing of the first horn but in the second transposing attempt flushing the other horn was not achieved. Considering only the ewes that had the cervix visualized and adequately retracted, results show there was 72.7% (8/11) in G-6 and 100% (9/9) in G-9 of ewes successfully flushed (two uterine horns flushed). The percentage of successful recoveries in G-9 was almost double that in the G-6 treatment group (Table 3).

Although the number of rings did not differ ($P > 0.05$) between treatments, the cervical misalignment score was higher ($P < 0.05$) in G-6, increasing ($P < 0.05$) at the time of transcervical penetration in this group compared to the G-9. There was no effect of treatment ($P > 0.05$) on the time of uterine flushing or for the total time of the embryo recovery procedure (Table 3). The time spent on cervical transposition did not differ ($P > 0.05$) between animals presenting a cervix with six or fewer rings (6.1 ± 1.0 min) and those with more than six rings (4.0 ± 1.0 min).

The number of recovered structures and number of blastocysts tended to be higher ($P = 0.07$) in G-9 and the number of viable embryos was higher ($P < 0.05$) in this treatment compared to the G-

Table 1
Ovulatory follicular dynamic and estrus end points (LSMEANS ± SEM) in Lacaune ewes subjected to estrus induction protocols* for six (G-6) or nine (G-9) days during the non-breeding season.

End points	G-6 days	G-9 days	P-value
Emergence day of ovulatory antral follicular wave[a] (days)	−3.0 ± 0.5	−4.2 ± 0.5	0.10
Duration of follicular growth phase of the largest preovulatory follicle (days)	5.1 ± 0.4	6.1 ± 0.4	0.11
Ovulatory diameter of the largest preovulatory follicle (mm)	6.9 ± 0.3 (13)	7.1 ± 0.3 (14)	0.66
Growth rate of the largest preovulatory follicle (mm/day)	0.9 ± 0.1 (13)	0.7 ± 0.1 (14)	0.13
Duration of follicular growth phase of the second-largest preovulatory follicle (days)	4.3 ± 0.5 (10)	5.0 ± 0.5 (13)	0.25
Ovulatory diameter of the second-largest preovulatory follicle (mm)	6.4 ± 0.3 (10)	6.1 ± 0.3 (13)	0.47
Growth rate of the second largest-preovulatory follicle (mm/day)	0.8 ± 0.1 (10)	0.7 ± 0.1 (13)	0.26
Estrus duration (h)	25.3 ± 2.6 (12)	21.5 ± 2.2 (12)	0.27
Interval from sponge removal to estrus onset (h)	36.7 ± 3.3 (12)	37.2 ± 3.4 (12)	0.91
Interval from sponge removal to ovulation (h)	57.1 ± 4.7 (13)	54.9 ± 4.3 (14)	0.74
Interval from estrus onset to ovulation (h)	21.9 ± 2.0 (13)	22.8 ± 2.0 (14)	0.75
Number of ovulations	2.0 ± 0.2 (13)	2.9 ± 0.3 (14)	0.02
% of multiple ovulations (≥3 CL)	21.4 ± 11.0 (14)	64.3 ± 12.8 (14)	0.03

( )Number of animals.
*Protocol with medroxyprogesterone acetate (60 mg) sponge plus 37.5 μg of d-cloprostenol and 400 IU of eCG 24 h before sponge removal.
[a] Relative to Day 0 (day of MAP sponge removal).

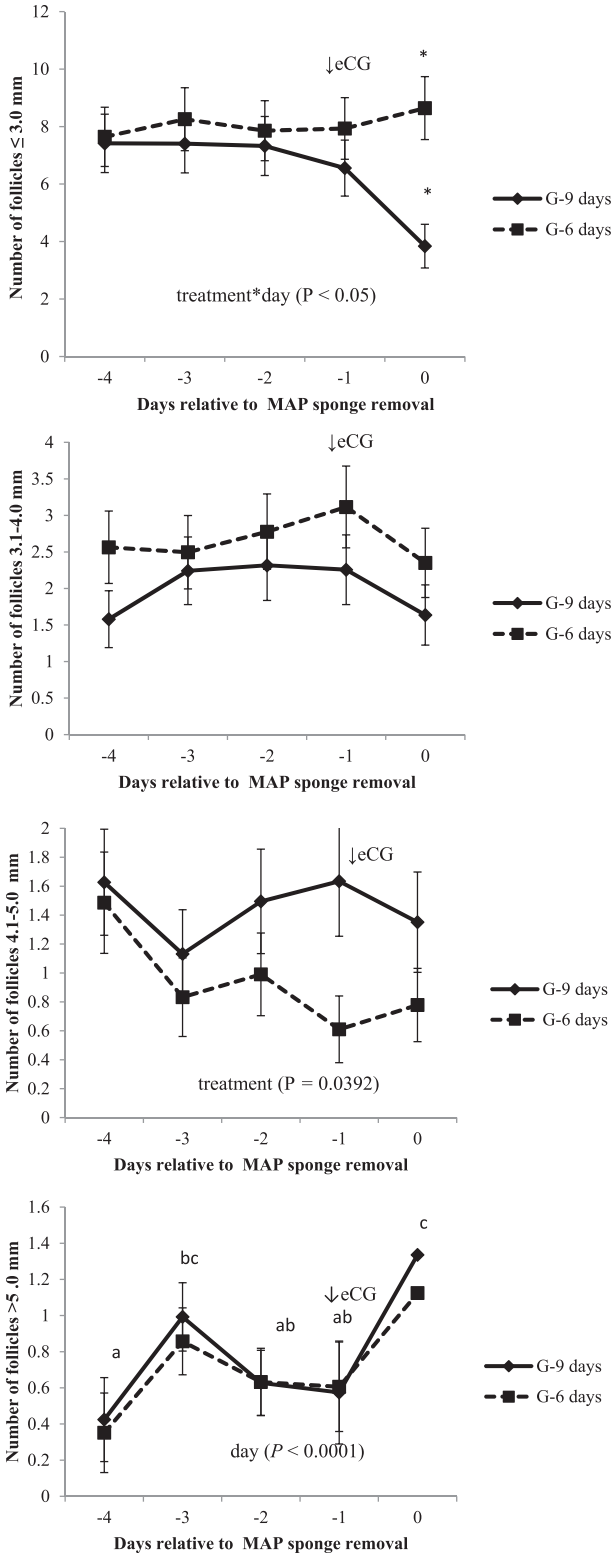

Fig. 2. Follicular counts (LSMEANS ± SEM) grouped by diameter categories (≤3.0 mm, 3.1–4.0 mm, 4.1–5.0 mm, > 5.0 mm) on the relative days to MAP sponge removal (Day 0) in Lacaune ewes subjected to synchronized estrus induction protocols with medroxyprogesterone acetate sponge for six (G-6, discontinuous line ----) or nine (G-9, continuous line ——) days plus 37.5 μg of d-cloprostenol and 400 IU of eCG 24 h before sponge removal.* Each asterisk indicates the difference between treatments ($P < 0.05$). [ab]Values with different letters differ between days ($P < 0.05$).

6. The number of morulae, the number of unfertilized oocytes, the unfertilized oocytes rate, and the viable embryo rate did not differ between treatments ($P > 0.05$, Table 4). The efficiency of embryos collected with the transcervical method was measured by the recovery of fluid and structures (recovery rate). The fluid recovery rate was 99.7% in G-6 and 99.9% in G-9. Recovery rates in G-6 and G-9 were 30.4% (7/23) and 48.3% (14/29), respectively. All recovered embryos (100.0%) were classified as Grade I in both treatments.

After embryo recovery, all ewes walked back to their pens and immediately searched for water or food. No ewe presented any uterine abnormality at ultrasonography performed 30 days after embryo recovery.

4. Discussion

The ability to set or adjust estrus induction/synchronization protocols according to the breed, animal category and condition of cyclicity of females would be favorable for increasing outcome efficiency, as well as to increase cost-benefits and to extend the use of this reproductive biotechnology in commercial herds. In addition, the ability to perform NSER by transcervical route in all sheep breeds would be extremely beneficial from both an ethical and practical perspective. The approach used in this study, i.e. to apply an estrus induction treatment, is a non-expensive way of testing transcervical penetration after cervical dilation treatment, as we have also carried out in other breeds [19,29].

The majority (57.1%) of Lacaune ewes had functional CL at the beginning of the estrus induction protocols. This indicates that a large percentage of the animals may still be cyclic in a tropical climate during the period of lengthening daylengths [15]. A similar percentage (56.5%) of Santa Inês ewes with CL was previously observed during the same climate and photoperiod [15]. Cyclicity in approximately half of Lacaune ewes raised at a high latitude and temperate climate, in Switzerland [2], suggesting that this breed is not strictly photoperiod-dependent, as previously reported in the lower latitude of Greece [30].

The emergence day of ovulatory follicular wave occurred on Day −3 (range −6 to −1) in G-6 and Day −4 (range −7 to −1) in G-9. However, it was observed wide range among ewes in both treatment groups. The progesterone treatment is not originally designed to synchronize follicular waves, however, there is a distinctive pattern of antral follicular wave dynamics during the treatment period, affected mainly by the number of emerging follicular waves and ovarian status at the beginning of the protocols [15]. This factor may justify the variations observed among the animals. In the Santa Inês breed, the emergence of the first and second antral follicular waves occurs approximately on Days 2.0 and 5.9 in relation to the CIDR insertion (Day 0), respectively [15]. If the antral follicular wave emergence pattern is the same for sheep of the Santa Inês and Lacaune breeds, this may indicate that the G-6 and G-9 ewes had follicle ovulation from the first and second antral follicular waves, respectively. Hormonal strategies to synchronize follicular wave emergence in sheep have not been as effective as reported in cattle. The use of P_4 devices associated with cloprostenol at the beginning of the protocol has been demonstrated as appropriate to synchronize follicular emergence (on average 56.6 h after device insert) in Santa Inês ewes without any benefit from adding GnRH agonists or estradiol benzoate [14]. In the present study, the d-cloprostenol dose was only administrated 24 h before MAP sponge removal, but perhaps if applied at the start of treatment this could reduce the variation for the follicular wave emergence day.

The fluctuation in the number of antral follicles, according to the size categories observed in the last five days of the protocols, correspond to the development of the ovulatory follicular wave. This reflects the progressive growth of small follicles from one size

Table 2
The number of corpora lutea, total luteal tissue area, vascularized total luteal area and percentage of vascularized luteal tissue area (LSMEANS ± SEM) on Day 5 after ovulation in Lacaune ewes subjected to synchronized estrus induction protocols[a] for six (G-6) or nine (G-9) days during the non-breeding season.

End points	G-6 days (n = 13)	G-9 days (n = 14)	P-value
Corpora lutea (n)	2.0 ± 0.2	2.9 ± 0.3	0.02
Total luteal tissue area (cm^2)	1.5 ± 0.2	2.2 ± 0.2	0.02
Vascularized luteal area (cm^2)	0.4 ± 0.1	0.6 ± 0.1	0.20
% of vascularized luteal tissue area	29.0 ± 4.0	29.0 ± 3.0	0.94

[a] Protocol with medroxyprogesterone acetate sponge plus 37.5 μg of d-cloprostenol and 400 IU of eCG 24 h before sponge removal.

Table 3
End points of cervical transposition and uterine flushing (LSMEANS ± SEM) in Lacaune ewes submitted to treatment to induce cervical dilation before non-surgical embryo recovery (NSER), performed 5–6 days after ovulation in response to synchronized estrus induction protocols* for six (G-6) or nine (G-9) days during the non-breeding season.

End points	G-6 days	G-9 days	P-value
Number of rings of cervix transposed	6.5 ± 0.8 (11)	6.3 ± 0.8 (9)	0.84
Successful recoveries[a] (%)	45.5 (5/11)	88.9 (8/9)	0.13
Degree of cervical misalignment[b]	2.3 ± 0.5 (11)	1.0 ± 0.3 (9)	0.01
Time of cervical transposition (min)	7.2 ± 1.3 (11)	3.4 ± 0.6 (9)	0.01
Time of uterine flushing (min)	18.3 ± 1.3 (8)	20.2 ± 1.3 (9)	0.34
Total time of embryo recovery procedure (min)	26.5 ± 2.2 (8)	23.8 ± 2.1 (9)	0.34

( )Number of animals.
*Protocol with medroxyprogesterone acetate sponge plus 37.5 μg of d-cloprostenol and 400 IU of eCG 24 h before sponge removal.
[a] Percentage of ewes in which at least one structure was recovered.
[b] Degree of cervical misalignment (one - rectilinear cervix, two - intermediate and three - highly asymmetrical).

Table 4
End points of embryo yield (LSMEANS ± SEM) in Lacaune ewes subjected to synchronized estrus induction protocols* for six (G-6) or nine (G-9) days and non-surgical embryo recovery (NSER), performed 5–6 days after ovulation.

End points	G-6 (n = 11)	G-9 (n = 10)	P-value
Recovered structures[a] (n)	0.6 ± 0.2	1.6 ± 0.4	0.07
Unfertilized oocytes (n)	0.2 ± 0.2	0.1 ± 0.1	0.45
Morulae (n)	0.1 ± 0.1	0.3 ± 0.2	0.27
Blastocysts (n)	0.3 ± 0.2	1.0 ± 0.3	0.07
Viable embryos[b] (n)	0.4 ± 0.2	1.3 ± 0.4	0.03
Viable embryo rate (%)	62.3 ± 0.2	87.7 ± 0.1	0.23
Unfertilized rate (%)	39.4 ± 23.9	6.5 ± 8.0	0.20

( )Number of animals.
*Protocol with medroxyprogesterone acetate sponge plus 37.5 μg of d-cloprostenol and 400 IU of eCG 24 h before sponge withdrawal.
[a] Structures definition: non-fertilized oocytes, embryos of different developmental stages and/or zona pellucida.
[b] Viable embryos were considered embryos Grade I, II and III.

class to another in a wave-like fashion [31]. On the day of MAP sponge removal, the number of small follicles (≤3 mm) was lower in G-9, possibly because the ovulatory follicular wave emerged earlier in these ewes (i.e. at least one day earlier compared to G-6 ewes).

The higher number of follicles between 4.1 and 5.0 mm in the G-9 could perhaps be related to the expected lower concentrations of progestogen in the last days of this hormonal protocol compared to the G-6 treatment. It is known that serum progestogen concentrations decline over time [32] and could fall to subluteal values after the 6th day [8,33]. A reduction of 50% was observed from the third day on the serum MAP concentrations in ewes treated over a long-term period with 60 mg MAP sponges outside the breeding season, which suggests that a limited quantity of progestogen is absorbed by the epithelium or that the clearance rate of the serum MAP is high [21]. These authors observed that ewes treated with halved sponges (30 mg) had a fecundity increase when compared to 60 mg. In ewes, the clearance of progesterone is increased from elevated feed intake [34]. Due to the high dry matter intake of the experimental animals, necessary to achieve nutrient requirements for milk production, it is possible to suggest that a high clearance rate may have been exacerbating subluteal conditions. Therefore, lower concentrations of progestogens possibly meant less suppression of endogenous LH concentrations, which could favor the development of a greater number of follicles in the dominant phase in G-9 compared to those ewes in G-6. This may also justify the higher number of ovulations in the G-9 ewes due to the lower dominance effect. It is known that the LH pulse frequency increases in the absence of a CL and that progestogen release is decreased over time [35,36], but the LH surge does not occur during treatment [8]. The gradual increase of LH pulsatility and/or the increase in serum FSH concentrations at proestrus are possibly responsible for follicle ovulations coming from the two last waves of prolific breeds [37]. The ovulation percentage coming from the last two waves in the G-9 was increased (~36%) and close to that observed by these authors (~50%). Dairy Lacaune is considered a low prolific breed [38], and the ovulation rates of prolific selected parents and the non-prolific are 2.35 and 1.67, respectively [39]. In double ovulatory cycles, the ovulatory follicles emerge as part of the same follicular wave but, in a few cases, also as part of different waves [8]. The higher frequency of ovulatory follicles from distinct waves in the G-9 was in some cases associated with multiple ovulations (triple, quadruple, and even quintuple) which, in turn, must have occurred due to the lower dominance effect. Lassoued et al. [40] also observed a higher number of medium follicles on the days prior to synchronized estrus in prolific strains when compared to non-prolific strains. Therefore, the G-9 protocol with eCG administered 24 h before sponge removal appears to mimic the hormonal and ovarian events that occur in prolific sheep breeds, so it seems to have a positive effect on ovulation rate in lactating Lacaune ewes.

Both hormonal treatments were efficient in inducing synchronous-estrus and promoted ovulation in a high percentage of females, similar to observed results in Lacaune ewes after P_4 treatments for 6 or 12 days in high latitude [2]. The duration of progesterone-based estrus induction protocols had no effect on the time of estrus and ovulation. The highest number of ovulations was responsible for the greater total luteal tissue area in the G-9 ewes compared to those from G-6, however, the characteristics of luteal

vascularization (i.e. vascularized luteal area and percentage of the vascularized luteal area), indicative of the luteal angiogenesis, were not altered by the MAP permanence. It is known that the production and release of progesterone are dependent on an active angiogenic process that occurs during the first few days after ovulation [41] and that both the total luteal area and progesterone concentration in sheep are positively correlated to each other between days 3 and 15 after ovulation [42]. In ewes, both the luteal area and the vascularized luteal area were positively correlated with progesterone during luteogenesis [25], suggesting that G-9 ewes possibly had higher progesterone concentrations.

This is the first report on NSER in Lacaune ewes. The transcervical penetration at the initial luteal phase after estrus synchronization provides a reliable screening test to determine the procedure efficiency and to select donors for the MOET program [19]. The cervical dilation protocol based on estradiol, oxytocin, and cloprostenol was efficient in Lacaune ewes, allowing for uterine flushing and embryo recovery in a high percentage of animals with ~100% of fluid recovery rate. Despite the use of a lower oxytocin dose, compared to Masoudi et al. [43], the effectiveness was greater (measured as the % of transcervical penetration) and may reflect previous exposure to a higher dose of estradiol benzoate and different physiological or anatomical conditions between breeds. Estradiol has several effects that may contribute to the regulation of uterine PGF2α secretion, maximizing its responsiveness to oxytocin and possibly modulating the pulsatile secretion of neurohypophyseal oxytocin [44].

It has been well established that changes in progesterone:estradiol ratio are important in the physiological and morphological changes of the cervix observed during parturition [45]. The effectiveness of estrogen being greater after a period of progesterone priming [46] – which promotes an accumulation of arachidonic acid, prostaglandin-endoperoxide synthase, and other molecules necessary for the synthesis of $PGF_{2\alpha}$in the endometrium [44] – ultimately downregulates its own receptor, allowing for estrogen-stimulated expression of oxytocin receptors [46]. These effects of progesterone appear to ensure that secretion of $PGF_{2\alpha}$ occurs at the appropriated time of induced luteolysis [44]. Therefore, an adequate progesterone profile prior to the cervical dilation treatment seems necessary for efficient NSER.

It can be supposed that an expected increase in circulating oxytocin in the group with more CL and luteal tissue (G-9) may play an important role in the lower cervical misalignment after cervical retraction and mean a shorter time for transcervical penetration, suggestive of better cervical dilation. The CL contains large amounts of mRNA and the protein for oxytocin synthesis and their concentrations stored in secretory granules reach their peak between days 5 and 9 after estrus [47], declining thereafter [48]. Thus, the application of d-cloprostenol before NSER may have induced luteolytic cascade and led to a greater release of oxytocin secretory granules by luteal cells in G-9 treatment. This synergic effect of exogenous and endogenous hormones seems to be important for NSER technique efficiency beyond the cervical anatomy. The physiology of cervical dilation depends on stimulating PGE synthesis in response to concentrations of reproductive hormones [49].

Recovery rates (~40.4%) in the present study are similar to those observed in non-superovulated Santa Inês ewes (41.2%) [19] and lower than the 65% observed in superovulated animals [50,51] by the transcervical method. The reasons for the low recovery rates observed in non-superovulated ewes are unclear. However, it seems to involve CL health status because, in ewes with <1 ng plasma progesterone concentrations at the time of collection, the embryo recovery rate was only 20% vs 50% in ewes with >1 ng [19]. In the current study, the percentage of successful recoveries (at least one structure recovered) was almost doubled in the group with the higher ovulation rate. Another study conducted by our team found similar (~65%) recovery rates in Lacaune superovulated ewes (article in preparation). Indeed, there are apparent limitations for achieving high rates of embryo recovery by the transcervical method in non-superovulated animals.

A higher number of recovered structures and viable embryos was found in the G-9 group, probably due to the higher number of ovulations. The percentage of viable embryos was 100% for both groups, suggesting that there was no deleterious effect from hormonal treatment to cervical dilation on embryo quality. This finding agrees with Figueira et al. [52], who observed good pregnancy and lambing rates after the transfer of frozen-thawed embryos retrieved by NSER (using the same cervical dilation treatment).

5. Conclusions

In conclusion, both synchronous-estrus induction treatments showed a high rate of estrus response and ovulation, but MAP sponge for 9 days increased the number of follicles between 4.1 and 5.0 mm and promoted a higher number of ovulations and embryo yield. Finally, the protocol combining d-cloprostenol, estradiol benzoate and oxytocin allowed for a high percentage of transcervical penetration followed by NSER in Lacaune ewes.

Authors' contributions

LMF developed the experimental activities, analyzed the data and wrote the manuscript; GNS and RRL analyzed the data; NGA, MEFO, and JMGSF corrected the manuscript; JFF designed and coordinated the study.

Declaration of competing interest

The authors declare that there is no conflict of interests.

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