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Repeated trans-cervical embryo recoveries in Santa inês ewes subjected to short- or long-term superovulatory treatment regimens

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ABSTRACT

Outcomes of short- (6.5 days) and long-term (14.5 days) estrous synchronization for 6.5 d (G-6.5d) or 14.5 d (G-14.5d) and followed by the 4-day or 3-day declining-dose follicle-stimulating hormone superovulatory regimen, respectively, were compared using 16 estrous-cycling Santa Inês ewes. Non-surgical embryo recovery (NSER) procedures were performed 60 d apart starting 6 or 7 d after the onset of estrus; an i.m. injection of estradiol benzoate and of D-cloprostenol at 16 h was followed by an i.v. oxytocin injection administered 20 min before NSER. There was a longer ($P < 0.05$) period before estrous onset in ewes during the second (September) compared with the first study replicate (July) by approximately 14 h. The NSER could be performed in 11 of 15 ewes that were in estrus, with an average of three viable-embryos/donor and the mean duration of the procedure being 29 min. There were no differences in superovulatory responses between the two groups of ewes, but there were only degenerated embryos in ewes of the G-6.5d group. In summary: i. the duration of progestin-priming and of multiple-dose pFSH treatment had a limited effect on superovulatory responses in estrous-cycling Santa Inês ewes; ii. NSER is a safe and repeatable method of embryo collection in ewes subsequent to superovulation; and iii. duration of the superovulatory treatment regimen may alter the effects of endogenous steroids on oocyte/embryo quality in ewes.

1. Introduction

The multiple ovulations and embryo transfer (MOET) program is a widely used technology to increase genetics that are considered elite from individual donor females. Presently available hormonal superovulatory procedures, however, are arduous. The superovulatory treatment of sheep and goats usually entails imposing estrous synchronization treatment regimens using vaginal delivery of progesterone/synthetic progestin for 12–14 d and administration of multiple doses of exogenous follicle-stimulating hormone (Fonseca et al., 2007). It has also been suggested that prolonged treatments with progesterone-releasing devices, typically associated with less than mid-luteal phase concentrations of biological actions induced by endogenous progesterone concentrations

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after approximately 10 d of treatment (Letelier et al., 2009), may negatively affect subsequent fertilization and early embryonic development processes (Gonzalez-Bulnes et al., 2005). In small ruminants, long-term progesterone pre-treatments result in a shorter interval to estrus when compared with when there is imposing of short-term treatment regimens (Harl, 2014), and the timing of estrus and of the preovulatory LH peak can affect superovulatory response (Veiga-Lopez et al., 2008). Even though both the short-term (5–7 d in duration) and long-term (12–14 d) progestin-based estrous synchronization regimens can be used to effectively while the follicular super-stimulation protocols are being imposed, there has only been one study directly comparing the effectiveness of two approaches in the ewes located in a subtropical habitat (Oliveira et al., 2014).

Follicle-stimulating hormone (FSH), a primary choice for hormonal ovarian superstimulation, is typically injected twice a day at 8-h or 12-h intervals over 3–4 d, starting 2 or 3 d before the removal of progestin-containing vaginal devices, respectively (Bartlewski et al., 2016). Decreasing doses of FSH are generally preferred because the imposing of this treatment regimen appears to result in an increase ovulatory responses and embryo viability rates in ewes where there has been super-stimulation of ovarian follicular development (Gonzalez-Bulnes et al., 2000). With the varying durations of progesterone priming-treatments described previously in this manuscript, the outcomes, as a result of ovarian follicular super-stimulation using the 3-d compared with 4-d multiple-dose FSH regimens in ewes, have not been compared in a single study.

There is considerable evidence indicating trans-cervical embryo recovery can be an efficacious alternative to or even completely replace the use of the surgical embryo flushing in ewes having normal estrous cyclic patterns when there are treatments to induce superovulations (Fonseca et al., 2019a). Recently, there has been use of a combination of treatment with estradiol benzoate, d-cloprostenol and oxytocin that induces cervical dilation, which allows for non-surgical embryo recovery and transfer in ewes (Fonseca et al., 2019b). The use of this technique still requires additional evaluations in various breeds of sheep as well as in animals undergoing different treatments to induce superovulation responses. There have not been evaluations of cervical penetration rates for embryo recoveries and the ease with which the uterine cervix can be traversed where there have been treatments to induce superovulation in ewes that are subjected to short- compared with long-term estrous synchronization treatment regimens. In addition, the effects of recurrent cervical dilation treatment regimens using estradiol benzoate, D-cloprostenol and oxytocin on the efficiency of embryo recovery have not been assessed.

The present study, therefore, was designed to compare the ovarian responses and embryo production in Santa Inês ewes subjected to either short-term (6.5 d) or long-term (14.5 d) progestin-based estrous synchronization treatment regimens and 4-d or 3-d decreasing-dose pFSH superovulatory treatments, respectively. The imposing of the short-term treatment regimens is associated with a delayed onset of estrus (Harl, 2014), indicating the occurrence of a delayed and/or longer period of antral follicular maturation. Embryos were recovered using trans-cervical uterine flushing procedures combined with SOV treatments and embryo recovery was performed at the 60-d intervals, so effects of concurrent ovarian stimulations were also assessed in the present study.

2. Materials and methods

2.1. Animals and superovulatory treatments

This study was approved by the Animal Care Committee of Embrapa Dairy Cattle (protocol 15/2014) and was conducted during a period from July to September (period of increasing day lengths characterized by the occurrence of recurrent estrous cycles in 50%–70% of ewes; Oliveira et al., 2016) at the Experimental Campus of Embrapa Dairy Cattle, in the rural area of Coronel Pacheco, MG, Brazil (latitude 21°35′S, longitude 43°15′W and altitude of 435 m.a.s.l.). A total of 16 multiparous, non-lactating Santa Inês ewes (body condition score of 4.2 ± 0.5; range: 1 - very thin to 5 - obese) were fed corn silage and *Pennisetum purpureum* as forage, with a balanced concentrate rationing (200 g/ewe/day). Mineralized salt blocks and drinking water were available *ad libitum*.

All animals were subjected to two ovarian follicular super-stimulation treatments to induce superovulation (Fig. 1) performed 60 d apart, in a cross-over experimental design; they were divided into the two equinumerous groups and allocated to either the G-6.5d or the G-14.5d group during the first study replicate (Replicate #1), and then to the other group 60 d later (Replicate #2). The ewes were fitted with intravaginal sponges containing 60 mg of medroxyprogesterone acetate (MAP; Progespon®, Schering Plough, São Paulo, SP, Brazil) that remained intra-vaginally for 6.5 day (G-6.5d) or 14.5 days (G-14.5d). The ewes in the G-6.5d were administered two i.m. injections of 37.5 μg of D-cloprostenol (synthetic analog of $PGF_{2\alpha}$; Prolise®, Tecnopec, São Paulo, SP, Brazil), one at the time of MAP sponge insertion and another at the time of sponge removal. The treatments for induction of superovulation began on Day 4 (G-6.5d) or Day 12 (G-14.5d) and consisted of eight or six i.m. injections of decreasing pFSH doses (Folltropin®-V, Bioniche Animal Health Canada Inc., Belleville, ON, Canada) administered every 12 h with a total amount of 200 mg of pFSH per ewe being used. All ewes were administered a single i.m. injection of 200 IU of equine chorionic gonadotropin (eCG; Novormon®, Syntex, Buenos Aires, BA, Argentina) at MAP sponge removal. To synchronize the timing of the preovulatory LH surge and ovulation among ewes, 0.025 mg of gonadotropin-releasing hormone (GnRH; Gestran®, Tecnopec, São Paulo, SP, Brazil) i.m. was administered 36 h after MAP sponge removal. Twelve hours after MAP sponge removal, all ewes were relocated to a pen with rams fitted with crayon-marking harnesses for the 3 d (rams to ewe ratio of 1:4). Rams used in this study underwent routine breeding soundness evaluation and were all classified as satisfactory. An i.m. dose of 200 IU of human chorionic gonadotropin (hCG; Vetecor®, Hertape-Calier do Brasil Ltda, São Paulo, SP, Brazil) was administered 2.5 d before embryo collection (i.e., 84 h after the onset of behavioral estrus; Saraheia, 1998).

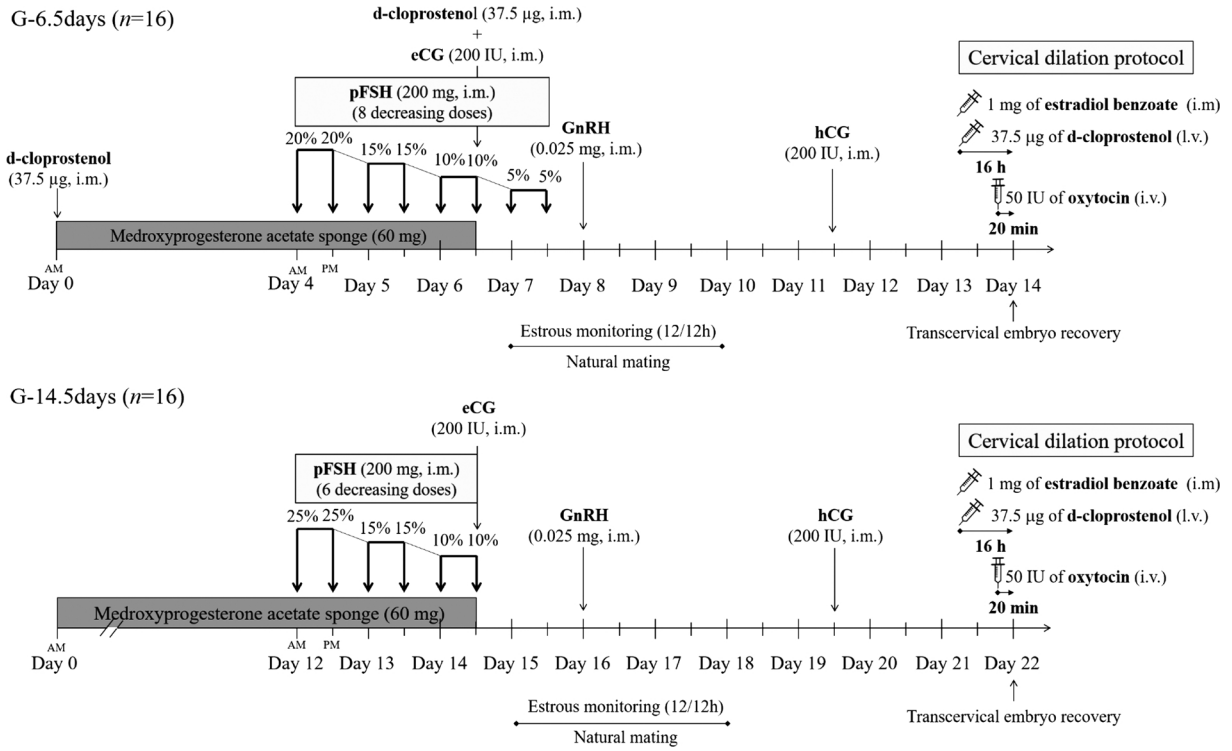

Fig. 1. Schematic depiction of the experimental procedures including the estrous synchronization protocols, superovulatory treatments, and cervical dilation protocol in Santa Inês ewes subjected to non-surgical embryo collection at 6 to 7 d after the onset of estrus; MAP: medroxyprogesterone acetate; eCG: equine chorionic gonadotropin; hCG: human chorionic gonadotropin; i.m. intramuscular; i.v. intravenous; l.v.: latero-vulvar.

2.2. Embryo collection by trans-cervical route

All ewes were administered i.m. injections of 1 mg estradiol benzoate (Estrogin®; Farmavet, São Paulo, SP, Brazil) and 37.5 µg d-cloprostenol at 16 h, and 50 IU of oxytocin (Ocitocina forte®; UCB, Jaboticabal, SP, Brazil) i.v. 20 min before uterine flushing for embryo collections. The embryo collections using the trans-cervical procedure was performed 6–7 d after the onset of behavioral estrus. Ewes were restrained in standing position and were administered acepromazine maleate (1 mg/kg i.m.; Aceproven®; Vencofarma, Londrina, PR, Brazil) 10 min before and a 2-mL lidocaine 2% epidural block (S5-C1; Lidovet®, Bravet, Rio de Janeiro, RJ, Brazil) immediately before the insertion of a vaginal speculum. Additionally, a sterile gauze swab soaked with 5 mL of lidocaine 2% without vasoconstrictors was placed in the vagina ventrally to the cervical os. Cervical penetration and uterine flushing were conducted using procedures previously described for sheep (Fonseca et al., 2019b). Briefly, the cervix was immobilized and retracted using the Allis forceps (26 cm). The catheter for uterine flushing (No. 08; Sonda Embrapa® for goat/sheep embryo recovery; Embrapa, Brasília, DF, Brazil) equipped with a metal mandrel was used to facilitate the cervical passage. Each uterine horn was flushed with 180 mL of PBS injected in fractions of ~10 mL. Trans-cervical penetration attempts were subsequently categorized using the five-point scale: Grade 1 (very easy; less than 1 min to complete); Grade 2 (easy; between 1 and 3 min); Grade 3 (intermediate; between 3 and 7 min); Grade 4 (difficult; between 7 and 10 min); and Grade 5 (impossible or no complete passage achieved; Fonseca et al., 2019a). The flushing solutions was placed in Petri dishes and embryos were morphologically classified using a stereomicroscope (20 to 50x magnification) based on stage of development and quality characteristics using the International Embryo Transfer Society (IETS) criteria as morulae or blastocysts of Grades I to IV; with embryos of Grades I-III being regarded as viable or transferrable quality embryos.

2.3. Blood sample collection and hormone assays

Blood samples were collected using jugular venipuncture procedures into heparinized tubes from all ewes at the time of MAP sponge insertion (Day 0), immediately before the first pFSH injection (Day 4 or Day 12 for G-6.5d and G-14.5d groups, respectively), at the time of MAP sponge removal (Day 6.5 or Day 14.5 for G-6.5d and G-14.5d groups, respectively), and immediately before embryo collections commenced (Day 14 or Day 22 for G-6.5d and G-14.5d, respectively) for measurements of plasma progesterone (P_4) concentrations. Blood samples collected immediately before EB injections (i.e., 16 h before uterine flushing) and immediately before uterine flushing were used to quantify circulating estradiol (E_2) concentrations. Plasma was immediately separated by centrifugation at 1500 × *g* for 15 min and stored at –20 °C. Estradiol concentrations were quantified using a commercial

radioimmunoassay kit (ImmuChem™ Coated Tube, 17β- Estradiol CT, MP Biomedicals, LLC – Orangeburg, NY, USA). The assay sensitivity and intra-assay coefficient of variation were 10 pg/mL and 11 %, respectively. Cross-reactivity of the primary antiserum with estrone and estriol was 6.2 % and 1.2 %, respectively, and cross-reactivity with other estrogens, gestagens and androgens evaluated was < 0.01 % (cross-reactivity with estradiol benzoate was not determined). Progesterone concentrations were quantified using the solid phase radioimmunoassay kits (Beckman Coulter®, Immunotec, Marseille, France) according to the manufacturer's specifications, as previously validated in our laboratory (Oliveira et al., 2018). The assay sensitivity and intra-assay coefficient were 0.05 ng/mL and 10 %, respectively.

2.4. Data analyses

The following data were recorded for both groups of ewes: estrus response (number of ewes in estrus/number of treated ewes × 100 %); time of the onset of behavioral estrus (relative to the time of MAP sponge removal); duration of estrus; extent of difficulty of cervical penetration procedure (scale 1–5 according to the time required to complete the procedure: 1: ≤1 min; 2: > 1 min and ≤5 min; 3: > 5 min and ≤10 min; 4: > 10 min; and 5: not penetrated; Fonseca et al., 2019a); percentage of successfully penetrated cervices for embryo collections (number of ewes for which there was success for embryo collections/total number of ewes in which cervical penetration was attempted × 100 %); time taken to penetrate the uterine cervix (in minutes); total duration of uterine flushing (in minutes, from the insertion of speculum to catheter removal); flushing fluid recovery rate; number of all recovered structures (including viable and degenerated embryos as well as unfertilized oocytes); number of viable embryos (embryo grades I-III); embryo viability rates (number of viable embryos/total number of recovered structures x 100 %); plasma P_4 concentrations (ng/mL) immediately before MAP sponge insertion, first pFSH injection, MAP sponge removal and embryo collection; and plasma E_2 concentrations (pg/mL) immediately before EB injection and embryo recovery.

Statistical analyses were performed using the SAS software (Cary, NC, USA) and SigmaPlot® (Systat Software Inc.; San Jose, CA, USA). Two-way analysis of variance (ANOVA) and the Tukey test were used for parametric data comparisons if there was a significant main effect of Group, Replicate or the interaction, and simple linear regression was used for correlation analyses. Analyses of proportions were done using a χ^2-test utilizing the Brandt–Snedecor formula (Cochran and Cox, 1957). All results are expressed as means ± standard errors of the mean (SEM). Differences were considered to exist when $P < 0.05$.

3. Results

Plasma P_4 concentrations were greater ($P < 0.05$) in ewes of the G-6.5d group compared with those of the G-14.5d group at the time of first pFSH injection, MAP sponge removal and embryo collection (Table 1). The proportion of ewes with plasma P_4 concentrations > 1 ng/mL (mid-luteal phase concentration) was greater ($P < 0.05$) in ewes of the G-6.5d compared with G-14.5d group at the time of MAP sponge insertion and immediately before the time of embryo collection; at the time of embryo recovery (Table 1). There were no differences ($P > 0.05$) between the two groups of ewes in mean plasma E_2 concentrations (Table 1).

Only one ewe (from the G-6.5d group) had not expressed symptoms of behavioral estrus within 72 h of the medroxyprogesterone acetate (MAP)-soaked sponge withdrawal (Table 2) and, therefore, there was no further use of the ewe in the study. The mean duration of behavioral estrus (47.6 ± 2.7 h, range: 12–72 h) did not vary ($P > 0.05$) between the two groups of ewes or during the different time-periods of the study (Replicate #1. compared with Replicate #2) in the present study (Table 2). Overall, there was a longer period to onset of estrus ($P < 0.05$) in ewes during the second compared with those during the first of the two study replicates (Replicate #2 compared with Replicate #1) by approximately 14 h (Table 2). In both study replicates, behavioral estrous occurred later in the ewes of the G-6.5d compared with the G-14.5d group (22.3 ± 1.7 compared with 16.0 ± 2.0 and 34.5 ± 3.4 compared with 30.8 ± 3.6, for Replicate #1 and Replicate #2, respectively). Of the ewes that expressed symptoms of behavioral estrus, the cervices of four ewes could not be penetrated when there were attempts to collect embryos [one animal could not be penetrated

Table 1
Mean (± SEM) values for plasma concentrations of progesterone (P_4) and estradiol (E_2) determined in multiparous, non-lactating Santa Inês ewes subjected to a 6.5-day (short-term; G-6.5d) or 14.5-day (long-term; G-14.5d) medroxyprogesterone acetate (MAP)-based estrous synchronization treatment regimen, multiple-dose pFSH treatment and cervical dilation treatment regimen prior to trans-cervical embryo collection performed twice, at a 60-d interval (Replicates 1 and 2), 6 to 7 d after the onset of estrus.

End points	G-6.5d	G-14.5d	*P* values	Replicate 1	Replicate 2	*P* values
	Plasma P_4 concentrations (ng/mL)					
at MAP sponge insertion	1.5 ± 0.4 (7/16)[a]	0.6 ± 0.2 (2/16)[b]	0.05	0.8 ± 0.3 (3/16)	1.3 ± 0.4 (6/16)	0.32
at 1st pFSH injection	0.5 ± 0.0 (0/16)	0.3 ± 0.1 (1/16)	0.03	0.4 ± 0.1 (1/16)	0.4 ± 0.0 (0/16)	0.91
at MAP sponge removal	0.4 ± 0.0 (0/16)	0.2 ± 0.0 (0/16)	< 0.0001	0.3 ± 0.0 (0/16)	0.3 ± 0.1 (0/16)	0.56
at embryo collection	3.3 ± 0.5 (15/16)[a]	1.0 ± 0.2 (6/16)[b]	0.0006	2.1 ± 0.6 (11/16)	2.2 ± 0.4 (10/16)	0.93
	Plasma E_2 concentrations (pg/mL)					
at EB injection	29.5 ± 4.3	21.7 ± 4.9	0.24	29.7 ± 6.3	21.9 ± 2.3	0.24
at embryo collection	220.5 ± 25.4	230.5 ± 16.8	0.82	220.2 ± 23.3	226.9 ± 32.0	0.86

Note: Proportion of animals with plasma P_4 concentration > 1 ng/mL are given in parentheses; Value of a single animal enclosed in brackets.
*Time from MAP sponge removal to the onset of estrus.
[a−b]Within rows different letters indicate differences ($P \leq 0.05$).

Table 2
Results (means ± SEM) of estrous detection, uterine flushing procedures and embryo yields in multiparous, non-lactating Santa Inês ewes subjected to two consecutive trans-cervical embryo recovery treatment regimens (Replicates 1 and 2) following a 6.5-day (short-term; G-6.5d) or 14.5-day (long-term; G-14.5d) medroxyprogesterone acetate (MAP)-based estrous synchronization treatment regimen, multiple-dose pFSH treatment and cervical dilation protocol prior trans-cervical embryo collection performed twice, at a 60-d interval (Replicates 1 and 2), 6 to 7 d after the onset of behavioral estrus.

End points	G-6.5d	G-14.5d	P values	Replicate 1	Replicate 2	P values
Estrus response (%)	93.7	100	0.32	100	93.7	0.32
Time of the onset of estrus*	28.8 ± 2.6	22.5 ± 2.6	0.10	18.7 ± 1.5	32.8 ± 2.5	< 0.0001
Duration of estrus (h)	50.4 ± 3.9	45.0 ± 3.9	0.33	50.2 ± 3.3	44.8 ± 4.4	0.33
Cervical penetration-degree of difficulty **	2.5 ± 0.3	2.7 ± 0.3	0.73	2.7 ± 0.3	2.5 ± 0.3	0.73
Duration of cervical penetration (min)	6.4 ± 1.4	4.8 ± 0.8	0.31	5.7 ± 1.1	5.4 ± 1.1	0.82
Ewes successfully collected (%)	86.7 (13/15)	81.2 (13/16)	0.32	81.2 (13/16)	86.7 (13/15)	0.32
Duration of uterine flushing (min)	29.1 ± 1.4	27.8 ± 1.4	0.54	28.1 ± 1.4	28.8 ± 1.4	0.70
Fluid recovery (%)	97.2 ± 0.5	95.5 ± 1.5	0.28	96.1 ± 1.6	96.5 ± 0.4	0.80
Ewes with recovered structures (%)	84.6 (11/13)	92.3 (12/13)	0.32	92.3 (12/13)	84.6 (11/13)	0.32
Number of recovered structures	5.8 ± 1.3	7.0 ± 2.0	0.61	8.4 ± 1.8	4.4 ± 1.3	0.08
Number of viable embryos (grades I-III)	2.9 ± 1.0	4.1 ± 1.3	0.49	3.7 ± 1.0	3.3 ± 1.3	0.82
Number of degenerated embryos (grade IV)	0.5 ± 0.2	0.0 ± 0.0	0.04	0.2 ± 0.2	0.8 ± 0.4	1.00
Number of unfertilized eggs	1.6 ± 0.7	2.7 ± 1.6	0.55	3.3 ± 1.6	0.8 ± 0.4	0.11
Embryo viability rate (%)	46.3 ± 12.3	56.2 ± 11.6	0.55	47.1 ± 10.2	65.6 ± 13.6	0.28

Note: *Time from MAP sponge removal to the onset of estrus.
**Scale of 1–5 based on the time required to complete the procedure (1-less than 1 min; 5-not penetrated).

during either of the two replicates (allocated to G-6.5d and G-14.5d, respectively); two ewes could not be penetrated only during Replicate #1 (both G-14.5d group), and one ewe could not be collected only during Replicate #2 (G-6.5d group)]. The cervical penetration score averaged 2.6 ± 0.2 [range: 1 (less than 1 min) to 5 (impenetrable)], average duration of cervical penetration procedure was 5.6 ± 0.8 min (range: 1–17 min), average duration of the uterine flushing was 28.5 ± 1.0 min (range: 21–40 min), and mean fluid recovery post-flushing was 96.3 ± 0.8 % (range: 78.5–100%; $P > 0.05$; Table 2). Furthermore, there were no differences ($P > 0.05$) in the number of all recovered structures (mean: 6.4 ± 1.2, range: 0–25) nor the number of viable embryos (mean: 3.5 ± 0.8, range: 0–13) and unfertilized eggs (mean: 2.1 ± 0.9, range: 0–21) collected per ewe after trans-cervical uterine flushing in both subsets of ewes, and degenerated embryos (numbering 0.5 ± 0.2 per ewe; range: 0–2) were only detected in the G-6.5d group for both study replicates ($P < 0.05$; Table 2).

In the ewes of the G-6.5d group, the number of degenerated embryos was positively correlated with plasma P_4 concentrations at the time of MAP sponge removal ($r = 0.58$, $P = 0.04$), first pFSH dose ($r = 0.59$, $P = 0.03$), and embryo recovery ($r = 0.56$, $P = 0.05$; Table 3). Circulating P_4 concentrations at the time of embryo collection were also directly related to the total number of recovered structures ($r = 0.56$, $P = 0.05$). In addition, embryo viability was positively correlated with plasma estrogen concentrations at the time of embryo collection ($r = 0.66$, $P = 0.04$) and negatively correlated with the duration of interval from MAP sponge removal to the onset of behavioral estrus ($r = —0.66$, $P = 0.02$). In the G-14.5d group, the duration of behavioral estrus was positively correlated with the number of viable embryos ($r = 0.56$, $P = 0.05$) and embryo viability rate ($r = 0.58$, $P = 0.04$). During the first study replicate, the mean duration of estrus was correlated with the number of viable embryos ($r = 0.60$, $P = 0.03$) and embryo viability rates ($r = 0.58$, $P = 0.04$) and plasma P_4 immediately before embryo collection was directly related to the number of viable ($r = 0.82$, $P = 0.0007$) and degenerated ($r = 0.56$, $P = 0.05$) embryos. During the second replicate conducted 60 d later, embryo viability rate was negatively correlated with the time of the onset of estrus ($r = —0.60$, $P = 0.05$) and P_4 concentrations at MAP sponge removal ($r = —0.62$, $P = 0.04$). Overall (i.e., with data for both groups and study periods combined), the mean duration of estrus ($r = 0.47$, $P = 0.02$) and estrogen concentrations at embryo recovery ($r = 0.57$, $P = 0.02$) were positively correlated with embryo viability rates and P_4 concentrations at embryo collection were directly related to the number of degenerated embryos ($r = 0.43$, $P = 0.03$).

4. Discussion

Results from quantification of circulating P_4 concentrations in the ewes of the present study revealed that 28 % of animals were in the luteal phase of the estrous cycle at the time of MAP sponge insertion. These results are not completely consistent with those of a previous study by Balaro et al. (2014) where it was reported that a small percentage of Santa Inês ewes had recurrent estrous cycles between July and September when located in a subtropical and tropical climate of Brazil. A difference in the percentage of estrous cyclic ewes between the G-6.5d (43.7 %) and G-14.5d (12.5 %) group appears to be coincidental because all ewes were randomly assigned to one of the two treatment groups. In the present experiment cloprostenol injections were administered at the time of MAP sponge insertion, which should have negated any effects of the difference in plasma P_4 concentrations between the two groups at least at the time of sponge removal. Even though there were lesser P_4 concentrations throughout the period of MAP treatment, plasma P_4 concentrations were greater in the ewes of the G-6.5d than in G-14.5d group on the day of first pFSH dose, sponge withdrawal and

Table 3
A list of significant correlations among estrous responses, plasma concentrations of steroid hormones and superovulatory responses in Santa Inês ewes following a 6.5-day (short-term; G-6.5d) or 14.5-day (long-term; G-14.5d) medroxyprogesterone acetate (MAP)-based estrous synchronization treatment regimens, multiple-dose pFSH treatment and cervical dilation protocol prior trans-cervical embryo collection performed 6 to 7 d after the onset of behavioral estrus.

Input variable (x)	Output variable (y)	r	*P* value	Regression equation
G-6.5d				
Onset of estrus*	Viability rate	—0.63	0.02	y = 119.6—2.0x
P_4 at MAP sponge removal (ng/mL)	No. of degenerated embryos	0.58	0.04	y = —0.5 + 3.2x
P_4 at first pFSH injection (ng/mL)	No. of degenerated embryos	0.59	0.03	y = —0.8 + 3.6x
P_4 at embryo collection (ng/mL)	No. of recovered structures	0.56	0.05	y = 3.9 + 1.3x
P_4 at embryo collection (ng/mL)	No. of degenerated embryos	0.56	0.04	y = —0.1 + 0.2x
Estrogens at embryo collection (pg/mL)	Viability rate	0.66	0.04	y = —14.5 + 0.3x
G-14.5d				
Duration of estrus (h)	No. of viable embryos	0.56	0.05	y = —1.1 + 0.05x
Duration of estrus (h)	Viability rate	0.58	0.04	y = —32.4 + 1.4x
Replicate 1				
Duration of estrus (h)	No. of viable embryos	0.60	0.03	y = —4.3 + 0.2x
Duration of estrus (h)	Viability rate	0.58	0.04	y = —32.9 + 1.5x
P_4 at embryo collection (ng/mL)	No. of degenerated embryos	0.82	0.0007	y = —0.2 + 0.2x
P_4 at embryo collection (ng/mL)	No. of viable embryos	0.56	0.05	y = 1.8 + 0.9x
Replicate 2				
Time of the onset of estrus	Viability rate	—0.60	0.05	y = 155.0—3.0x
P_4 at MAP sponge removal (ng/mL)	Viability rate	—0.62	0.04	y = 121.9—238.1x
Overall				
Duration of estrus (h)	Viability rate	0.47	0.02	y = -12.0 + 1.3x
P_4 at embryo collection (ng/mL)	No. of degenerated embryos	0.43	0.03	y = —0.05 + 0.1x
Estrogens at embryo collection (pg/mL)	Viability rate	0.57	0.02	y = —9.7 + 0.3x

Note: r-coefficient of correlation;
*Time from MAP sponge removal to the onset of estrus.

embryo recovery. Even though in both treatment groups circulating P_4 concentrations after cloprostenol injections were less than typical mid-luteal phase concentrations of the hormone, the differences in P_4 concentrations are unexpected and difficult to explain. It is important to note that medroxyprogesterone acetate does not cross-react with the primary antibodies used in our radioimmunoassay of endogenous progesterone. When P_4-impregnated siliconized devices were used in goats (Souza et al., 2011) and sheep (Pinna et al., 2012), however, P_4 from both endogenous and exogenous sources could be quantified but specific amounts from the two sources could not be ascertained using radioimmunoassays. Furthermore, medroxyprogesterone acetate mimics the effects of endogenous P_4, suppressing LH secretion and consequently luteal P_4 synthesis and release. This results in a progressive decrease in plasma P concentrations in both goats (Fonseca et al., 2008) and sheep (Calvacanti et al., 2012) pretreated with progestin-containing intravaginal devices. It is feasible that animals subjected to the short-term treatment with MAP-releasing sponges in the present study had a larger number and/or greater P_4 secretory capacity of the corpora lutea (CL) compared with the ewes of the G-14.5d group even though there was a D-cloprostenol injection on the day of MAP sponge insertion; although the ovulation rate was not recorded, the number of animals with plasma P_4 concentrations < 1 ng/mL at the time of embryo collection was greater ($P < 0.05$) in ewes of the G-14.5d than in G-6.5d (10/16 compared with 1/16, respectively) group. Furthermore, all ewes were treated with an injection of hCG early in the luteal phase to prevent premature luteal regression and/or to stimulate the formation of accessory CL as described by Saharrea et al. (1998). This treatment, however, seems to have been more effective in the ewes of the G-6.5d group than those of the G-14.5d subset of animals.

The period of behavioral estrus in the ewes of the present study began ~6 h later in the ewes of the G-6.5d than those in G-14.5d group; this difference was mainly apparent during the first study replicate (data not shown) but overall was not a difference from a statistical perspective. Notably, the onset of behavioral estrus occurred ~14 h later during the second (September) compared with the first (July) study replicate. Short-term estrous-synchronization treatment regimens using P_4 or synthetic progestins are associated with a delayed onset of estrus when compared with the long-term protocols (Harl, 2014), possibly due to the effects of exogenous P_4/progestins on FSH-stimulated antral follicle emergence and growth (Bartlewski et al., 2003, 2004; 2008; 2009); an increase in circulating P_4 concentrations results in an advanced first peak in FSH secretion but has no effect on the timing of subsequent peaks and follicular wave emergence. Because antral follicular waves emerge approximately every 4 d in ewes (Baby and Bartlewski, 2011a, b; Bartlewski et al., 2011, 2017), the growth of ovulatory follicles during the period of imposing the short-term estrous synchronization treatment regimen begins shortly before the cessation of treatment with exogenous P_4/progestin. Hence, in the present study, a pFSH treatment of ewes undergoing the 6.5-d synchronization was conducted for 4 consecutive days to extend the period of the effect to pFSH stimulation. Furthermore, because the ewes in the G-6.5d group had greater P_4 concentrations on the day of MAP sponge removal compared with the ewes in the G-14.5d group, the onset of estrus could have been delayed in the ewes of the G-6.5d group due to a slower rate of CL regression. Lastly, the second replicate of this study took place 60 d after the first one and the difference in mean day length between July and September exceeds 1 h. Even though sheep located in a subtropical climate are generally less sensitive to photoperiodic stimulation than the breeds maintained in temperate climates (Bartlewski et al., 2011), the effects of

photoperiod on the onset of sexual receptivity in the ewes of the present study cannot be completely discounted (Balaro et al., 2014).

Circulating P_4 concentrations recorded at different time points throughout the study period were associated with the numbers of degenerated embryos and total numbers of recovered structures only in the ewes of the G-6.5d group; circulating P_4 concentrations at the time of embryo recovery were positively correlated with the total number of recovered structures and plasma P_4 concentrations throughout the entire study period were directly related to the number of degenerated embryos. Those observations are consistent with the results of earlier studies using long-term follicular super-stimulation treatment regimens for superovulation in which circulating P_4 concentrations prior to embryo recovery had a limited effect on embryo viability in ewes (Bartlewski et al., 2008). Correlations observed in the G-6.5d group may simply reflect greater ovulatory responses; in ewes where there were treatments to induce superovulation, an increase in ovulation rate is frequently associated with a greater proportion of poor-quality embryos (Bartlewski et al., 2016, 2017). In the ewes of the G-6.5d group, embryo viability was also positively associated with the early onset of estrus and relatively greater estrogen concentrations at the time of embryo recovery. Both these correlations are difficult to explain. In the G-14.5d group, the mean duration of estrus was positively correlated with the number of viable embryos/embryo viability rate. Veiga-Lopez et al. (2008) reported that the shortened periods of behavioral estrus coupled with earlier occurrence of LH surges were associated with a larger number of degenerated embryos and lesser embryo viability rates in ewes in which there was treatments to induce superovulation. Interestingly, when correlation analyses were conducted separately for the two study replicates, the only two variables associated with embryo quality in Replicate #1 were the duration of estrus and P_4 concentrations at the time of flushing for embryo collections, whereas in Replicate #2, the time of the onset of estrus and its possible cause (P_4 concentrations at MAP sponge removal) were the exclusive correlates with embryo viability rates. The reason(s) for inconsistent correlations among circulating concentrations of steroids hormones, timing of the estrous period and embryo quality in ewes when there treatments to induce superovulation after the short- or long-term superovulatory regimen and at different times of the year remain(s) to be elucidated.

The 4-d ovarian super-stimulation treatment regimen may result in induction of an increase in follicular blood flow, which can have a negative effect on oocyte quality (Oliveira et al., 2014). Specifically, there was a positive correlation between the follicular blood flow on the fourth day of the treatment regimen and the number and percentage of unfertilized eggs in Santa Inês ewes (Oliveira et al., 2014). In the present study, there was no difference between the two groups of ewes in the number of embryos recovered, but there were only degenerated embryos collected from the ewes in the 4-d pFSH treatment group.

In small ruminants, trans-cervical embryo recovery is a less invasive alternative to the widely used uterine flushing technique using laparotomy (Fonseca et al., 2019a). A combined treatment with estradiol benzoate, D-cloprostenol and oxytocin induces enough cervical dilation to perform trans-cervical embryo collection in estrous-synchronized Santa Inês ewes (Fonseca et al., 2019b). The present experiment, however, is the first report regarding the application of cervical dilation treatment regimens in Santa Inês ewes where there were treatments to induce superovulation. The percentage of ewes where there was cervical passage of the tube used to collected embryos was ~90 %, the procedure was associated with a large percentage of flushing solution recovery (~ 96 %), and the total duration of uterine flushing was slightly less than 30 min. Results of the present study, therefore, are supportive of the efficacy of NSER for embryo recovery after superovulatory treatments of ewes. The NSER success rate for embryo recoveries in the present study was actually greater than those in a previous study conducted with the same breed of sheep when there was use of different treatment regimens for cervical relaxation (80 %; Gusmão et al., 2007). Furthermore, this method of embryo recovery can be successfully repeated in the same donor ewes after a 60-d interval indicating it is safe and does not cause cervical lesions that would preclude consecutive trans-cervical collections of embryos. The cervix of only one ewe could not be penetrated in both study replicates whereas the cervix in three other ewes could not be traversed only once, at the first or second replicate. These results indicate, at least in some cases, the same female may respond differently to the cervical dilation protocol applied at a 60-d interval. More studies are needed to determine the causes of such individual variations.

The effect of a combined treatment with estradiol benzoate (EB), D-cloprostenol and oxytocin on embryo viability remains unknown (Fonseca et al., 2019b), although there are reasonable pregnancy rates after fixed-time embryo transfer of frozen-thawed embryos collected from Lacaune donors using the same cervical dilation protocol that was used in the present study (Figueira et al., 2019). Treatment of the ewes with estrogens may have adverse effects on the preimplantation embryo (Flöter et al., 2018). In the present study, plasma concentrations of estrogens increased ~10-fold from the time of EB injection to embryo recovery. The endocrine milieu induced as a result of the cervical dilation treatment regimen did not alter the morphology of sheep embryos. The embryo viability rate was approximately 51 %, which is comparable to those reported in ewes that underwent surgical embryo collection (51.6 %, Oliveira et al., 2012; 37.5 %, Oliveira et al., 2014; 64 %, Bartlewski et al., 2015; 47 %, Bartlewski et al., 2017).

In a previous study conducted in Santa Ines ewes from March to June, laparoscopic embryo recovery was attempted 90 d apart only in ewes with an ovulation rate of ≥ 5; laparotomy and embryo recovery were not performed in 27 % (4/15) and 40 % (6/15) of the ewes after the first (May) and second (June) treatment to induce super-ovulations (Cordeiro et al., 2003). The total number of recovered structures and the number of viable embryos were 5.1 and 3.4 after the first or 3.6 and 2.4 after the second study replicate, respectively. There were similar results in the present study (Table 2) even though all ewes that were in estrus were subjected to NSER regardless of the ovulatory response; there was no determination of number of CL in the present study to simulate a “blind” embryo collection (Fonseca et al., 2013). In the present study, at least one structure was recovered with NSER in 85%–92% of donor ewes. Considering all the results related to NSER efficacy (percentage of ewes in which there were successful collections of embryos: 81.2 %–86.7 %; fluid recovery: 95.5 %–97.2 %; number of total structures and number of viable embryos recovered in Replicate #1 (8.4 and 3.7) and #2 (4.4 and 3.3), respectively), the outcomes from the present study was the most desirable when there was NSER performed in Santa Inês ewes as compared with outcomes in previous studies. Not only were the embryonic yields greater in the present study than those reported from a study using surgical embryo collection from the donor Santa Inês ewes with ≥ three functional CL (Pinto et al., 2020) but these results also indicate the suitability of the method of embryo recovery that can be

conducted in field conditions without previous food/water deprivation, anesthesia, costly equipment and procedures, and considerable stress endured by animals subjected to invasive procedures.

Circulating estrogen concentrations on the day of embryo recovery were positively correlated with embryo viability rates in the ewes of the G-6.5 group. These findings indicate that at the doses used in this study, a hormonal mixture including estradiol benzoate administered 16 h prior to embryo collection had no adverse effects on or even improved the quality of sheep embryos recovered. A reason(s) for a lack of a similar relationship in the ewes undergoing a long-term treatment regimen to induce super-ovulations is not known. Furthermore, developmental potential of embryos obtained using NSER procedures remains to be determined.

5. Conclusions

The results of the present study indicate that duration of treatment regimens to induce super-ovulation may potentially alter the effects that endogenous and exogenous steroids have on oocyte/embryo quality in ewes treated to induce super-ovulations. The duration of progestin priming and multiple-dose pFSH treatment, however, had a limited effect on embryo yields in Santa Inês ewes treated to induce super-ovulations approximately 60 d apart. Furthermore, non-surgical embryo recovery (NSER) is a safe and repeatable method of embryo collection in ewes induced to have super-ovulations resulting in a large rate of embryo recovery.

Author contributions

JFF conceived the present study; MEFO, JMGS-F, JDG and JFF finalized the experimental design; FNZ, JMGS-F and JFF collected the data from the animals; MEFO, JMGS-F, JDG, JFF and FZB performed initial analyses of the data; FZB performed hormone assays and hormonal data analyses; MEFO and PMB performed additional statistical analyses; MEFO wrote the first version of the manuscript; PMB revised it critically, and MEFO, FNZ, PMB, JDG, FZB, JMGS-F and JFF all approved of the final version of the paper.

Declaration of Competing Interest

Nothing to declare.

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