Animal Reproduction Science 223 (2020) 106644

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Luteotropic effects of human chorionic gonadotropin administered 7.5 days after synchronous estrous induction in Morada Nova ewes

Gabriel Brun Vergani [a], Jeferson Ferreira da Fonseca [b,c,*], Juliane Teramachi Trevizan [a], Verônica Schinaider do Amaral Pereira [d], Alexandre Rossetto Garcia [d], Sergio Novita Esteves [d], Felipe Zandonadi Brandão [e], Joanna Maria Gonçalves Souza-Fabjan [e], Maria Emilia Franco Oliveira [a,b,*]

[a] Department of Pathobiology and Veterinary Theriogenology, School of Agricultural and Veterinarian Sciences, São Paulo State University, Jaboticabal, SP, Brazil
[b] Embrapa Caprinos e Ovinos, Rodovia MG 133, Km 42, Cep 36.155-000, Coronel Pacheco, MG, Brazil
[c] Departamento de Veterinária, Universidade Federal de Viçosa, Av. Peter Henry Rolfs, s/n, CEP 36570-000, Viçosa, MG, Brazil
[d] Embrapa Pecuária Sudeste, Rodovia Washington Luiz, Km 234 s/n°, Fazenda Canchim, Caixa Postal: 339, CEP: 13560-970, São Carlos, SP, Brazil
[e] Faculdade de Veterinária, Universidade Federal Fluminense, Rua Vital Brasil Filho, 64, CEP 24230-340, Niterói, RJ, Brazil

ARTICLE INFO

Keywords:
Accessory corpora lutea
hCG
Luteal vascularization
Luteal blood flow
Ovarian ultrasonography
Progesterone

ABSTRACT

This study was conducted in ewes to assess effects of human chorionic gonadotropin (hCG) administration after imposing an estrous induction treatment regimen. Ewes (n = 115) were treated with a 60 mg medroxyprogesterone-intravaginal-sponge for 6 d plus 200 IU of equine chorionic gonadotropin (eCG) im and 37.5 μg d-cloprostenol im 36 h before sponge removal (Day 0). After natural mating, ewes having at least one corpus luteum (CL; n = 108) were administered either 1 mL of saline (G-Control; n = 53) or 300 IU of hCG (G-hCG; n = 55) on Day 7.5 after sponge removal (Day 0). Ovarian ultrasonography and blood collection were performed on Days 7.5, 13.5, 17.5, 21.5, and 30.5. Accessory CL (aCL) were observed in 81.5 % (G-hCG) and 0.0 % (G-Control) of ewes (P = 0.0001). Diameter, area, and volume of luteal tissue were greater (P < 0.05) in G-hCG from Day 13.5 to 30.5. Progesterone (P_4) concentrations were greater (P < 0.05) on Days 13.5, 17.5, 21.5 and 30.5 for ewes of the G-hCG group. Pregnancy percentage was similar (P = 0.25) between groups [47.1 % (G-control) compared with 60.0 % (G-hCG)], although total number of lambs produced by estrous synchronized ewes was greater (P = 0.005) in ewes of the G-hCG group (90.9 % compared with 66.0 %). In conclusion, hCG administration 7.5 days after sponge removal from Morada Nova ewes during the non-breeding season is an effective treatment to induce aCL formation, improve luteal tissue biometry and P4 concentrations, and to enhance the total number of lambs born.

* Corresponding author at: Embrapa Caprinos e Ovinos, Rodovia MG 133, Km 42, Cep 36.155-000, Coronel Pacheco, MG, Brazil.
E-mail addresses: jeferson.fonseca@embrapa.br (J.F. Fonseca), m_emiliafraoli@yahoo.com.br (M.E.F. Oliveira).

https://doi.org/10.1016/j.anireprosci.2020.106644
Received 8 May 2020; Received in revised form 15 October 2020; Accepted 16 October 2020
Available online 2 November 2020

1. Introduction

The early gestation period is a time when there can be pregnancy losses for numerous reasons, including non-infectious maternal factors such as a sub-optimal hormonal milieu (Rhind et al., 1978) and effects of hormones on the uterine milieu. Results from studies with ruminants indicate there is a positive and indirect effect of progesterone (P_4) on the uterine milieu and, consequently, on early embryo development and quality (Satterfield et al., 2006; Lonergan et al., 2016). Changes in the protein content of the uterine luminal fluid are associated with greater embryonic nutrition and improvement in the uterine conditions that are conducive for embryo viability (Forde and Lonergan, 2017).

The use of luteotropic hormones such as human chorionic gonadotropin (hCG) and gonadotropin-releasing hormone (GnRH) to induce the formation of accessory corpora lutea (aCL) and, consequently, to increase the P_4 concentration and the conception rate has been investigated in cattle (Fonseca et al., 2000, 2001; Maillo et al., 2014) and goats (Fonseca and Torres, 2005; Fonseca et al., 2006). Especially in sheep, results from several studies indicate hCG administration had effects on aCL formation and improved the P4 concentrations (Khan et al., 2009; Allam et al., 2015; Coleson et al., 2015; Fernandez et al., 2018; Fonseca et al., 2018). Even though there are these positive effects, there have been no improvements in pregnancy rate of ewes as a result of this treatment (Catalano et al., 2015; Fernandez et al., 2019). Factors affecting the most expected outcome – pregnancy rate increase – are still lacking. Nonetheless, the ovarian follicular characteristics at the time of gonadotropin administration and the P_4 threshold necessary for pregnancy establishment and maintenance have not yet been determined.

Studies in Santa Inês sheep in tropical conditions and subjected to a short-term treatment regimen had an interval from sponge removal to ovulation of 58 (Cavalcanti et al., 2012) and 70 (Teixeira et al., 2016) h. The first follicular wave of the estrous cycle emerges about the same time that ovulation occurs, and the dominant follicle persists for 9 days after ovulation in Corriedale ewes (Viñoles et al., 1999). The dominance state of the follicle probably occurs on Day 7–8 subsequent to ovulation. Formation of aCL was observed in 85.7 % of nulliparous Santa Inês ewes treated with 250 IU hCG on Day 8 (Fonseca et al., 2018) and 100 % of Merino ewes treated with 300 IU hCG on Day 7 (Fernandez et al., 2018), both after sponge removal. There were suggestions that only follicles $\geq$ 4 mm of diameter formed aCL; because these follicles already have LH receptors (Driancourt, 2001). Noteworthy, the follicular population present in the ovary on any specific day of the estrous cycle varies depending on the number of waves of follicular dynamic changes that occur for each ewe during the estrous cycle, generally three to four (Bartlewski et al., 2011). In ewes in which there are three or four waves of follicular dynamic changes follicles $\geq$ 4 mm in diameter generally develop around the eighth day of the estrous cycle (Evans et al., 2000). Ovulations from follicles that are of this size can occur with gonadotropin treatments with there subsequently being aCL formation.

Another important factor for the aCL formation to occur is the "health" of the dominant follicle on the day of gonadotropin administration. Ovarian follicular development and regression patterns were initially proposed (Menchaca and Rubianes, 2004) for dominant follicle development preceding ovulation in sheep. A more advanced stage of follicular development pattern during the estrous cycle was described for the largest follicles in goat does compared to what occurs in ewes (Carvalho-de-Paula et al., 2020). In these studies, there was a lesser probability for ovulations from regressing follicles in sheep, and in goats, the follicles in a more advanced stage of development were associated with abnormal CL functions subsequent to ovulations from the follicle in more advanced stages of development. The hormonal treatment regimen that is utilized for estrous cycle control, therefore, may affect whether there is favorable dominant follicular status for ovulation from a fully functional follicle after intravaginal device removal.

Considering these combined results, the working hypothesis for the present study was that administration of hCG 7.5 days after the estrous induction treatment regimen was imposed in Morada Nova ewes during the non-breeding season would induce 1) aCL formation, 2) an increase in dimensions (diameter, area, and volume) and functionality of luteal tissue (vascularization parameters and P4 concentration), and 3) have a positive effect on the pregnancy rate and number of multiple births.

2. Materials and methods

2.1. Ethics, animals and experimental design

The Animal Use Ethics Committee of EMBRAPA Southeast Livestock (#09/2017) approved this study. The experiment was conducted in a completely randomized block design, with three blocks starting 1 week apart, in October and November (non-reproductive season).

A total of 115 Morada Nova ewes [primiparous (n = 37) or pluriparous (n = 78)] clinically healthy, non-pregnant, non-lactating, with 36.6 $\pm$ 4.8 kg of body weight (BW) and 3.1 $\pm$ 0.2 of body condition score (BCS), on scale of 1–5 according to Villaquiran et al. (2007) were used. The ewes were managed in an intensive breeding system at the Canchim farm of EMBRAPA Southeast Livestock (21°57′28.5″ South and 47°50′36.6″ West), and were maintained in paddocks with there being rotations through different paddocks with cultivated pasture (*Panicum maximum* cv. Aruana). Ewes also had access to mineral mix and water *ad libitum*. The eses were fed nutritional supplements containing corn silage and a balanced nutrient supplement in amounts of 200 g/animal/day, provided once daily.

A device containing 60 mg of medroxyprogesterone acetate (MAP; Progespon®, Zoetis, USA) was inserted intravaginally and remained in place for 6 days with there being administration of 37.5 μg d-cloprostenol (Veteglan®, Hertape Calier, Barcelona, Spain) im and 200 IU of eCG (Novormon®, Zoetis, USA) im 36 h before sponge removal. Both sponge insertion and removal were performed between the 1700–1800 h. After sponge removal, ewes were allocated to be mated by four rams (ram:ewe 1:10 ratio). Rams had been previously evaluated for libido and seminal quality and remained in the paddock with ewes for 3 days. Ewes that had at least one CL on

Day 7.5 (*i.e.*, ewes that responded to estrous synchronization treatment regimen, $n = 108$) were allocated into one of two experimental groups, with stratification into groups based on BW, BCS, parity order, and ram. Females were subjected to the initial procedures of the treatment regimen and on Day 7.5 (7–8 h am) after sponge removal were administered either 300 IU of hCG (Vetecor®, Hertape-Calier) im (G-hCG, $n = 55$; BW: 36.3 ± 4.0 kg, BCS: 3.1 ± 0.3 and 2.5 ± 1.8 parity order) or 1 mL saline solution (0.9 % NaCl, Eurofarma Lab SA, Brazil) im (G-Control, $n = 53$; BW: 36.6 ± 5.5 kg, BCS: 3.1 ± 0.3 and 2.5 ± 1.6 parity order) (Fig. 1).

2.2. Ovarian ultrasonography and pregnancy diagnosis

Ovarian ultrasonographic evaluations (B-mode and Color Doppler) were performed in all experimental ewes ($n = 108$) on Days 7.5, 13.5, 17.5, 21.5 and 30.5 after sponge removal. A portable ultrasonic device equipped with a multifrequency linear transrectal transducer (6–8 MHz, My Lab Vet 30 Gold, Esaote, Netherlands) was used. The Color Doppler settings were maintained during all evaluations (Pulse repetition frequency (PRF): 1.4 KHz; Gain 64 %; Depth 8 cm; CFM (Frequency): 5 MHz; and Wall Filter (WF): M). The number of antral follicles on Day 7.5 and CL present on all days when there were evaluations were quantified and dimensions of the structures were determined. For this purpose, the sonographic plane of the section, which included the structures in largest structural dimensions, was selected for value determinations. The diameter was calculated by determining the average of two perpendicular measurements. The follicular population was classified into four categories according to diameters: total antral follicles ($\geq$ 2 mm), small ($<$ 3.5 mm), medium (3.5–4.49 mm), and large ($\geq$ 4.5 mm) follicles (Oliveira et al., 2016). Percentages of ewes with at least one follicle $\geq$ 3.5 mm, $\geq$ 4 mm, or $\geq$ 4.5 mm were determined (considering that 4 mm follicles have LH receptors; Driancourt, 2001). The CL area was ascertained by determining the structures outer perimeter utilizing the capacity of the ultrasonic equipment. The estimated CL volume was calculated using the sphere volume formula ($4/3 \times \pi \times r^3$), with r = radius and $\pi = 3.1416$. For cavitary CL, the dimensions of the cavity were subtracted. For all measurements, the sum of the CL present in ewes on the day of evaluation were calculated. The area of vascularization present in the CL was determined with the aid of the Image J® software in the number of colored pixels and later transformed into mm^2. The percentage of the luteal structures with vascularization was calculated using the formula: (number of colored pixels/number of total pixels of the CL) X 100. The value for combined CL structures was determined when it was not possible to ascertain the individual CL structure the average was calculated by the number of CL present. The vascularization volume was estimated using the formula (average of percentage of vascularization x summed volume of the luteal tissue/100).

Females that on Day 17.5 had CL with no vascularization (*i.e.*, considered the end of the luteal phase for ewes in which stage of cycle was synchronized) were not evaluated on Days 21.5 and 30.5. Pregnancy diagnosis was performed on Day 30.5 using B-mode ultrasonography of the uterus, confirming the presence of an embryonic vesicle and a fetal heartbeat. The ewes were monitored during the parturition periods to determine the number of lambs produced by each pregnant female. The lamb(s) BW was determined immediately after birth, and the litter BW at birth was also determined.

2.3. Blood sampling and serum P_4 concentration measurement

Blood samples were collected from 30 ewes ($n = 15$ per treatment, eight pregnant and seven non-pregnant each) on the day when all ultrasonic evaluations occurred (Days 7.5, 13.5, 17.5, 21.5, and 30.5), by jugular vein puncture into vacuum tubes without anticoagulant. At about 30 min subsequent to blood sample collection, the samples were centrifuged at 3000 X *g* for 10 min, and the serum was stored at -20 °C in two aliquots. The quantitation of serum P_4 concentrations were performed according to Fonseca et al. (2018), using the solid-phase radioimmunoassay (RIA) technique using a commercial kit (ImmuChem, MP Biomedicals, Santa Ana, CA, USA), following the manufacturer's recommendations. The average intra-assay sensitivity and coefficient of variation was 0.05 ng/mL and

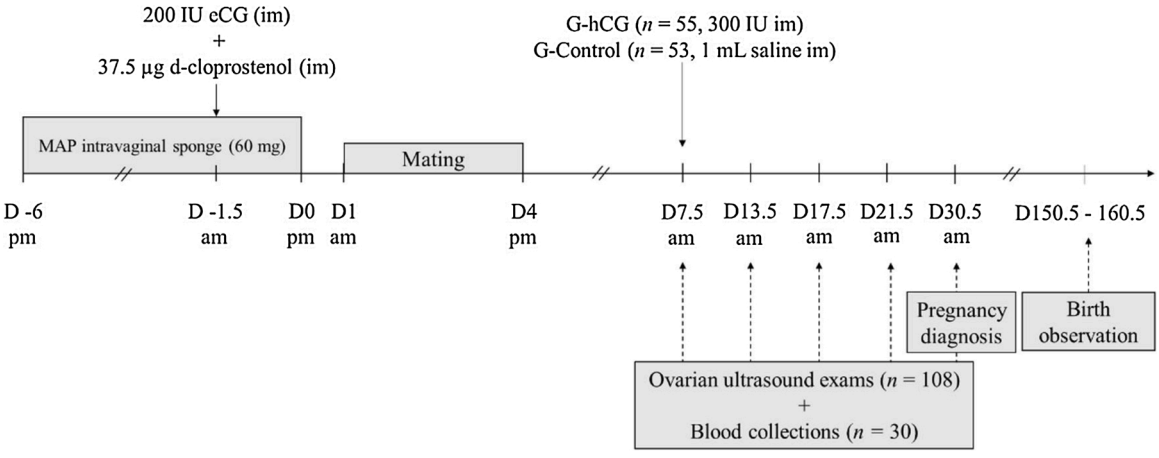

Fig. 1. Schematic representation of the experiment undertaken to evaluate luteotropic effects of administration of 300 IU of hCG 7.5 days after imposing a synchronous estrous induction treatment regimen in Morada Nova ewes during the non-breeding season.

11 %, respectively.

2.4. Variables and statistical analyses

The values for following variables were recorded: Number of antral follicles in different diameter categories; Percentages of ewes with at least one follicle ≥ 3.5 mm, ≥ 4 mm or ≥ 4.5 mm; Percentage of ewes with at least 1 aCL; Number of CL; Proportion of ewes with aCL; Sum of diameter (mm), area (mm^2) and volume (mm^3) of luteal tissue; Average percentage of tissue vascularization of luteal tissue (%); Sum of vascularized area of luteal tissue (mm^2); Pregnancy rate (number of pregnant females/number of females X 100) (%); Parturition rate (number of females lambing/number of pregnant females X 100) (%); Total pregnancy loss (number of females that did not produce a lamb/number of pregnant females) (%); Multiple birth rate (number of ewes producing at least two lambs/number of ewes that had parturitions X 100) (%); Total number of lambs born; Prolificacy (number of lambs born/number of ewes that produced lambs X 100) (%); Number of lambs born/number of ewes having a synchronized estrous cycle on which the treatment regimen was imposed X 100 (%); Lamb weight at birth (kg); Litter weight at birth (kg); and Serum P_4 concentration (ng/mL).

Analysis of data was performed using Statistical Analysis System software (SAS). The Cramer-Von Mises test was used to verify the normality and the homoscedasticity of the data. Data were transformed using the Box-Cox procedure when needed (Box and Cox, 1964), however, data are presented in the non-transformed state. Parametric data were analyzed with PROC MIXED procedure with a repeated statement to assess the interaction between sequential measurements. Non-parametric data were analyzed using PROC NPAR1WAY with the Wilcoxon test. Data were partitioned into two periods relative to the day of the luteal phase: (1) Days 7.5–17.5 (all ewes: both pregnant and non-pregnant) and (2) Day 17.5–30.5 (only data from pregnant ewes). In the former group (all ewes), the effects in the statistical model included treatments, days of evaluation, pregnancy diagnosis, and the interactions; for the latter (only pregnant), the effects included treatments, days of evaluation, and the interaction. The reproductive performance, number of CL, and follicular population were compared between groups using an one-way ANOVA, Fisher's Exact Test, or Chi-square test, and a *post-hoc* comparison was performed using the Tukey test. The Pearson correlation test was used to estimate associations between values for different variables. There were considered to be mean differences when there was a $P < 0.05$, and there was considered to be tendency for differences when there was a *P*-value between 0.051 and 0.10. The results in the text, tables, and figures are reported as means ± standard error of the mean (SEM).

3. Results

The values for antral follicular population present in the ovaries on Day 7.5 after sponge removal are reported in Table 1. Most (81.5 %) of the ewes of the G-hCG group had aCL compared with none (0.0 %) of the ewes in the G-control group. On Day 7.5, the percentage of ewes with follicles ≥ 3.5 mm and ≥ 4.5 mm tended to be greater ($P = 0.08$) in ewes of the G-Control group. The number of total antral follicles (≥ 2 mm) and small antral follicles (< 3.5 mm) was greater ($P < 0.03$) in ewes of the G-Control group. The number of large follicles (≥ 4.5 mm) tended to be greater ($P = 0.057$) in ewes of the G-Control group. There was no correlation ($P > 0.05$) between the values for antral follicular population (number of total, small, medium, and large follicles and diameter of the largest follicle) with the number of aCL formed.

For ewes of the G-hCG group, the values for comparison of the ovarian follicular population on Day 7.5 in ewes either having or not having an aCL are reported in Table 2. The percentage of ewes with follicles ≥ 4.0 mm was greater ($P = 0.01$) in ewes with aCL. The number of small antral follicles (< 3.5 mm) was greater ($P = 0.01$) in ewes without aCL, while the number of medium-sized follicles tended to be greater ($P = 0.07$) in ewes with aCL.

For the luteal dimensions (diameter, area, and volume of luteal tissue) and vascularized dimensions (volume and area of vascularized luteal tissue), there were interactions ($P < 0.003$) between the values associated with effects of treatments, pregnancy diagnosis and days of evaluation from Days 7.5 to 17.5. From Days 17.5 to 30.5, there was only a treatment effect ($P < 0.01$; Fig. 2A–E). For values of CL number and P_4 concentration, there were interactions between treatments and days of evaluation ($P < 0.01$) and between pregnancy diagnosis and days of evaluation ($P = 0.03$) from Days 7.5 to 17.5. In contrast, the treatments were the only effect ($P = 0.007$) from Days 17.5 to 30.5. For percentage of vascularized luteal tissue, there was only a difference between the time of pregnancy

Table 1
Mean ± SEM of the antral follicular population present in the ovaries in Morada Nova ewes treated with either 300 IU of hCG (G-hCG) or 1 mL of saline solution (G-Control) on Day 7.5 after imposing a synchronous estrous induction treatment regimen in the non-breeding season.

Variables	G-Control	G-hCG	*P*-value
Rate of ewes with aCL formed (%)	0.0 (0/53)	81.5 (44/55)	< 0.0001
Ewes with follicles ≥ 3.5 mm (%)	94.3 (50/53)	81.8 (45/55)	0.08
Ewes with follicles ≥ 4.0 mm (%)	77.35 (41/53)	61.8 (34/55)	0.12
Ewes with follicles ≥ 4.5 mm (%)	60.3 (32/53)	41.8 (23/55)	0.08
Nb of total follicles (≥ 2 mm)	5.8 ± 0.2	4.5 ± 0.2	< 0.0001
Nb of small follicles (< 3.5 mm)	3.7 ± 0.2	2.8 ± 0.2	0.002
Nb of medium follicles (3.5–4.49 mm)	1.3 ± 0.1	1.2 ± 0.1	0.52
Nb of large follicles (≥ 4.5 mm)	0.6 ± 0.1	0.4 ± 0.1	0.057
Diameter of the largest follicle (mm)	4.5 ± 0.1	4.3 ± 0.1	0.30

*aCL: Accessory corpora lutea.

Table 2
Values for variables (mean ± SEM and %) according to antral follicular population present in the ovaries of Morada Nova ewes treated with 300 IU of hCG on Day 7.5 after imposing a synchronous estrous induction treatment regimen in ewes during non-breeding season that developed or did not develop accessory corpora lutea (aCL).

	G-hCG		
Variables	Without aCL	With aCL	*P*-value
Ewes with follicles ≥ 3.5 mm (%)	63.6 (7/11)	86.3 (38/44)	0.18
Ewes with follicles ≥ 4.0 mm (%)	27.2 (3/11)	70.4 (31/44)	0.01
Ewes with follicles ≥ 4.5 mm (%)	27.2 (3/11)	45.4 (20/44)	0.32
Nb of total follicles (≥ 2 mm)	4.9 ± 0.5	4.4 ± 0.2	0.65
Nb of small follicles (< 3.5 mm)	3.9 ± 0.6	2.5 ± 0.2	0.01
Nb of medium follicles (3.5–4.49 mm)	0.7 ± 0.3	1.3 ± 0.1	0.07
Nb of large follicles (≥ 4.5 mm)	0.2 ± 0.1	0.5 ± 0.1	0.24
Diameter of the largest follicle (mm)	3.9 ± 0.3	4.4 ± 0.1	0.20

diagnosis and days of evaluation ($P = 0.0001$) from Days 7.5 to 17.5 while there was only the effect of days of evaluation from Days 17.5 to 30.5 ($P = 0.0003$; Fig. 3A–C). The number of CL that developed as a result of hCG treatments increased from Day 7.5 to 13.5 and remained greater until Day 30.5 in ewes of the G-hCG group. In pregnant ewes, this variable was greater on Days 13.5 and 17.5 compared with non-pregnant ewes (Fig. 3A). The diameter, area, and volume of luteal tissue on Day 7.5 were similar ($P > 0.05$) between all subsets. The diameter and area of the luteal tissue increased ($P = 0.003$) from Day 7.5 to 13.5 in all ewes of the G-hCG group, regardless of ewe pregnancy status; and only in pregnant ewes, were these variables greater until Day 17.5 (Fig. 2A–B). The volume of luteal tissue increased ($P = 0.0007$) from Day 7.5 to 17.5, only in pregnant ewes of the G-hCG group (Fig. 2C). In pregnant ewes, from Day 17.5 to 30.5, the diameter, area, and volume of luteal tissue were greater in ewes of the G-hCG group (Fig. 2A–C).

The ewes of the G-hCG group had P_4 concentrations that increased ($P < 0.001$) from Day 7.5 to 13.5 ; similarly, ewes that were pregnant of the G-hCG group had greater P_4 concentrations from Day 17.5 to Day 30.5 compared to ewes of the G-control group. Regardless of the treatment, in both pregnant and non-pregnant ewes, the P_4 concentrations increased from Day 7.5 to 13.5 and, in pregnant ewes, remained greater on Day 17.5 (Fig. 3B). For the percentage of vascularization of the luteal tissue, there was a difference between the interaction of pregnancy diagnosis and days of evaluation: non-pregnant ewes had the least values ($P = 0.0001$) on Day 17.5 (Fig. 3C). The vascularized area of luteal tissue increased ($P < 0.01$) from Day 7.5 to 13.5 in both pregnant and non-pregnant ewes from the G-hCG group. In contrast, the vascularized volume of luteal tissue was greater only in pregnant ewes of the G-hCG group. There were greater values for both variables of vascularization on Day 17.5 compared with other subsets. From Day 17.5 to Day 30.5, there were greater values in the ewes of the G-hCG compared with G-Control group (Fig. 2D–E). The values for all biometric variables of functional luteal tissue were positively correlated ($P < 0.02$), except for the number of CL with the percentage of luteal vascularization and the latter with volume of luteal tissue (Table 3). The values for percentage of luteal vascularization were correlated least closely with the other endpoints.

The pregnancy rate did not differ ($P = 0.25$) among groups (Table 4), however, pregnancy rate was greater ($P = 0.03$) in ewes of the G-hCG group with aCL (70.4 %, 31/44) compared with those without aCL (27.3 %, 3/11) or ewes of the G-Control group (47.1 %, 25/53). There was a positive correlation ($P < 0.01$) between the proportion of ewes with aCL development and the pregnancy rate ($r = 0.34$). For the reproductive performance indices (Table 4), the number of lambs born/number of ewes with a synchronized stage of the estrous was greater ($P = 0.003$) in ewes of the G-hCG group.

4. Discussion

This is the first report of aCL induction in sheep with detailed assessment using color Doppler ultrasonography of the ovaries. The most important findings were that hCG administered 7.5 days after the end of the estrous induction treatment regimen was effective for inducing aCL development, increasing the luteal tissue biometry (number of CL, area, and volume) and functionality of these tissues (P_4 concentration). There were larger numbers of total and small follicles on Day 7.5 in ewes of the G-Control group, therefore, it is logical to assume that there would have been greater aCL development in ewes of the G-hCG group if there would have been similar follicle populations in this group. Furthermore, it is suggested that the follicular cells of follicles ≥ 3.5 mm may have been converted into luteal tissues as a result of the hCG administration and thus there would have been development of aCL. The pregnancy rate was 23.3 % greater in ewes of the G-hCG group in which there was aCL development compared with ewes of G-Control group. Furthermore, the number of lambs born/number of ewes on which the estrous synchrony regimen was imposed was greater (24.9 %) in ewes of the G-hCG group.

The day of hCG administration in the present study was selected based on the expected time of ovulation after the treatment regimen for estrous synchronization was imposed in the present study (Cavalcanti et al., 2012) and ovarian follicular wave emergence (Viñoles et al., 1999) reported in other sheep breeds because such information was not available for Morada Nova ewes. The large aCL development rate in the present study (81.5 %) was similar to the 85.7 % reported in Santa Inês breed (Fonseca et al., 2018) and indicates that the time of hCG administration was adequate. The similarity between the percentage of ewes developing aCL and the percentage of ewes with follicles ≥ 3.5 mm in ewes of the G-hCG group indicates the theca and granulosa cells of the follicles of this size may have developed into luteal tissues of aCL. This possibility is supported by the trend ($P = 0.07$) for a greater number of medium follicles (3.5–4.49 mm) for ewes with aCL in hCG-treated group. This possible effect likely was enhanced as a result of the relatively

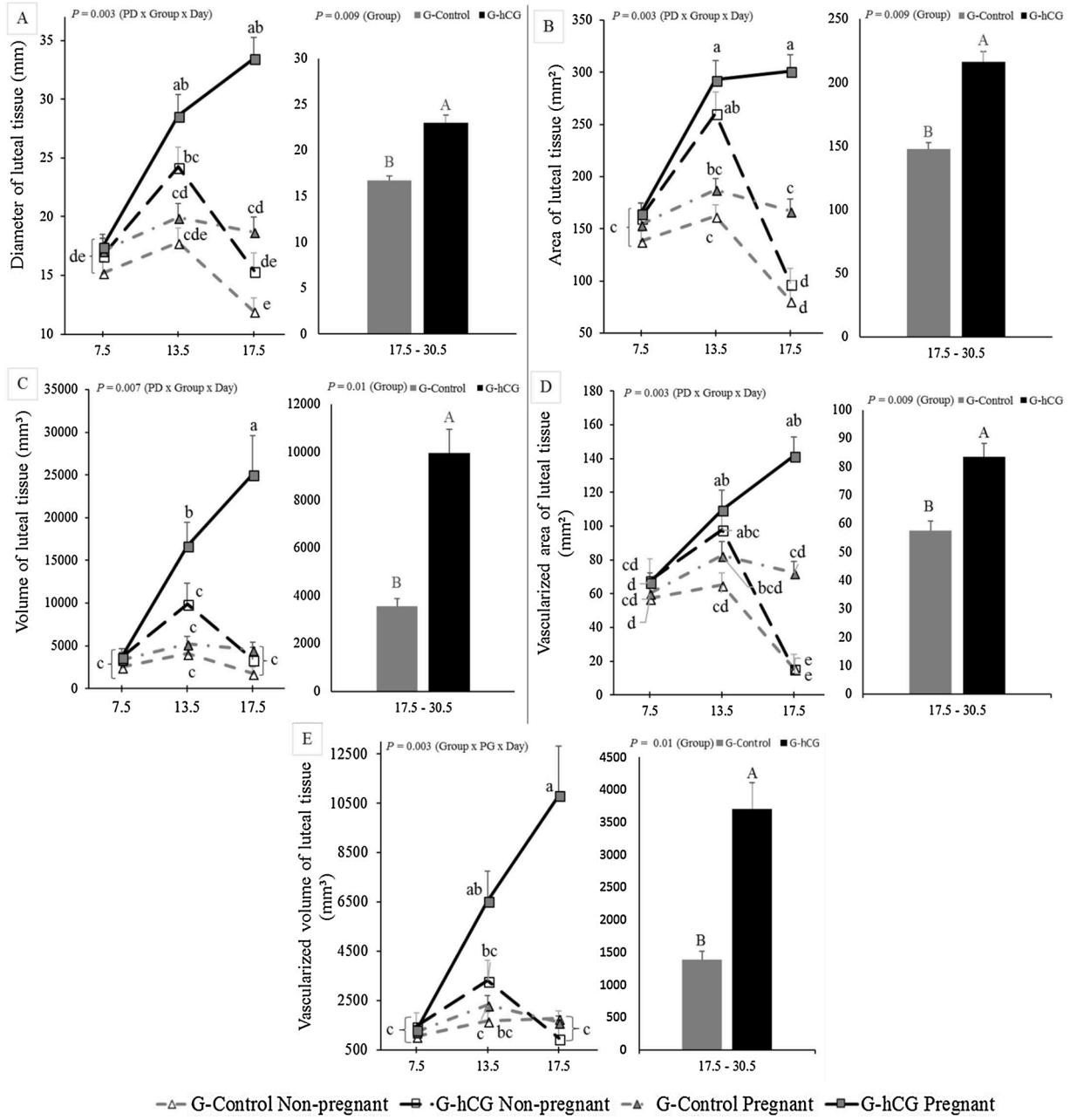

Fig. 2. Mean (± SEM), diameter (A), area (B), volume (C) of luteal tissues, vascularized area (D) and vascularized volume (E) of the corpus luteum on Days 7.5, 13.5, 17.5, 21.5 and 30.5 of non-pregnant and pregnant Morada Nova ewes administered 300 IU hCG (G-hCG) or 1 mL of saline solution (G-Control) on Day 7.5 after imposing a synchronous estrous induction treatment regimen; *Different superscript letters indicate mean differences ($P < 0.05$); **PD = Pregnancy diagnosis.

long hCG half-life in circulation as compared with most other gonadotropins (Yen et al., 1968) and follicular growth rate of 0.6 mm per day (Viñoles et al., 1999). Fernandez et al. (2018) suggested that with administration of hCG there was development of aCL from follicles ≥4 mm, and it is known that LH receptors are present in the cells of 4 mm follicles (Driancourt, 2001). There, however, are no reports confirming the minimum diameter of the follicles that is necessary for these follicular cells to be converted into aCL as a result of gonadotropin treatment.

There are few studies of luteal dynamics in response to hCG treatment to induce aCL in ewes. These few studies were restricted to the evaluation of the CL area on specific days of the luteal phase (Fonseca et al., 2018) or biometrics (*e.g.*, weight and diameter) on a single day (post-mortem) (Khan et al., 2007; Allam et al., 2015; Catalano et al., 2015). In the present study, the administration of hCG on Day 7.5 resulted in an increase in the CL number and dimensions (diameter, area, and volume), as well as in the area and volume of

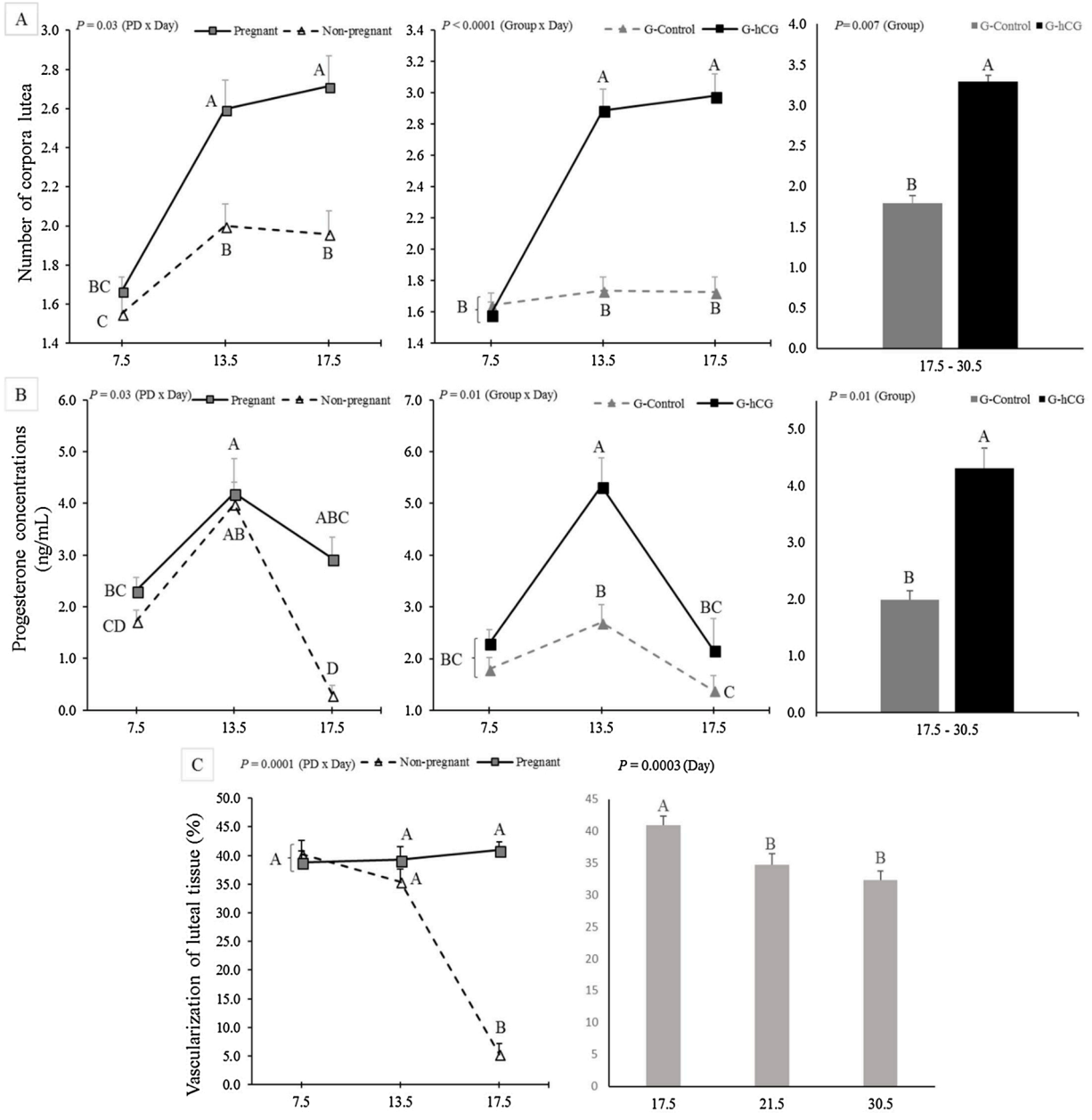

Fig. 3. Mean (± SEM) of the number of corpus luteum (A), serum progesterone concentrations (B), vascularized percentage (C) of luteal tissue on Days 7.5, 13.5, 17.5, 21.5 and 30.5 of non-pregnant and pregnant Morada Nova ewes treated with 300 IU hCG (G-hCG) or 1 mL of saline solution (G-Control) on Day 7.5 after imposing a synchronous estrous induction treatment regimen; *Different letters indicate mean differences ($P < 0.05$); **PD = Pregnancy diagnosis.

luteal vascularization and P_4 concentrations on Days 13.5, 17.5, 21.5 and 30.5, especially in pregnant ewes. These positive effects were expected and may be directly associated with aCL formation or as a consequence of the hCG luteotropic effect on the original CL, as reported in cattle (Schmitt et al., 1996; Binelli et al., 2001; Fonseca et al., 2001). The results of the present study do not allow for ascertaining whether the hCG action was exclusively induction of development of aCL or if there were also actions of hCG on the CL that developed after ovulation from the dominant follicle during the follicular phase of the estrous cycle. The P_4 concentrations, however, had greater coefficients of correlation with the dimensions of the luteal tissue ($r \geq 0.57$) than with the CL number ($r = 0.45$). It is believed, therefore, that there was also a luteotropic effect on the CL that developed after the ovulation during the follicular phase of the estrous cycle.

The increase in P_4 concentrations from Day 7.5 to 13.5 in ewes of G-hCG group also confirms there is an association between luteal biometry and potential for steroidogenesis, as reported to have occurred in an *in vitro* study (Veenhuizen et al., 1972). Similar results

Table 3
Pearson correlation coefficient (r) between the values for biometry and functionality variables of the luteal tissue of Morada Nova ewes with at least one accessory corpora lutea treated with 300 IU of hCG on Day 7.5 after imposing a synchronous estrous induction treatment regimen during the non-breeding season.

Variables	Number of CL	Diameter of luteal tissue	Area of luteal tissue	Volume of luteal tissue	Progesterone concentration	Percentage of luteal vascularization	Area of luteal vascularization
Diameter of luteal tissue	0.93	1					
Area of luteal tissue	0.84	0.92	1				
Volume of luteal tissue	0.84	0.91	0.81	1			
Progesterone concentration	0.45	0.59	0.70	0.57	1		
Percentage of luteal vascularization	–	0.16	0.20	–	0.46	1	
Area of luteal vascularization	0.68	0.75	0.76	0.67	0.67	0.61	1
Volume of luteal vascularization	0.78	0.86	0.77	0.93	0.56	0.30	0.80

*All the coefficients presented were significant ($P < 0.02$).

were reported from several *in vivo* studies (Khan et al., 2007; Lankford et al., 2010; Catalano et al., 2015; Coleson et al., 2015; Fernandez et al., 2018; Fonseca et al., 2018). Based on results from these numerous studies, there was a difference for the interval between treatment and increase of P_4 concentrations, which may be related to the dose used, days of treatment, breeds, and seasons. Apparently, there is an indirect correlation between the dosage of the gonadotropin and the beginning of the response. It is noteworthy that in the present study, the P_4 concentrations were greater during the time period when there was maternal recognition of pregnancy, from 12 to 13 days after estrus (Ford, 1985). In general, with greater P_4 concentrations, there are positive effects on the uterus as a result of optimal concentrations of specific proteins in the luminal fluids. This results in optimal conditions for nutrient availability to the embryo and accelerates development and favors uterine receptivity of the embryo (Forde and Lonergan, 2017).

Color Doppler ultrasonography is an effective method for studying tissue vascularization and associations with tissue functionality in sheep, particularly the follicular (Oliveira et al., 2014, 2017) and luteal (Figueira et al., 2015; Arashiro et al., 2018; Oliveira et al., 2018) tissues. The present report is thought to be the first in which there was applying color Doppler procedures for the study of aCL induction in sheep. As expected, the luteal vascularization (*i.e.*, area and volume) was greater in pregnant ewes that had been treated with hCG and was positively correlated with the functionality of luteal tissue. There are similar associations between the area of luteal vascularization and P_4 concentrations throughout the sheep estrous cycle (Figueira et al., 2015). In addition, the subjective assessment of the vascularization percentage is effective procedure for the early diagnosis of pregnancy in cattle (Pugliesi et al., 2014), sheep (Arashiro et al., 2018), and goats (Cosentino et al., 2018). The quantitative (non-subjective) determination of the percentage of vascularization of luteal tissue was not an effective approach for detecting effect of the hCG treatment on functional CL, although the timing of onset of luteolysis can be detected using this procedure, as previously reported by Figueira et al. (2015). The determination of the percentage of vascularization is possibly a less effective approach than assessments of the vascularized area and volume. This supposition is based on the fact that percentage vascularization determinations do not reflect the dimension of luteal tissue, which is associated with the capacity for P_4 synthesis (Schmitt et al., 1996).

Even though there all the positive effects of hCG on luteal dynamics of Morada Nova ewes, there was no effects on pregnancy and parturition rates when there was administration of hCG to induce development of aCL. These results corroborate results from previous studies in which there was evaluation of aCL induction in sheep (Khan et al., 2007; Catalano et al., 2015; Fonseca et al., 2018; Fernandez et al., 2019). Considering that the present study was conducted in ewes during the non-breeding season, results might have been different if there had been the treatment regimens imposed that were used in the present study during the breeding season. Improvements in the treatment regimen is needed to increase the percentage of aCL formation because there is a positive correlation between the formation of aCL and pregnancy percentage.

It is important to highlight a few factors that may have affected the pregnancy rate in the current study. A limitation in the present study, was the lack of determination of estrous and mating responses as result of imposing the treatment regimen. The total number of ewes used may also have been a limitation in evaluating pregnancy rates, because even with the 13 % greater pregnancy rate in ewes of the G-hCG treatment group, there was not a detectable difference in pregnancy percentages between groups. Future studies, therefore, are necessary, with a larger number of ewes, to effectively evaluate pregnancy rates when imposing the treatment regimens evaluated in the present study. Interestingly, the pregnancy rate was greater in the ewes treated with hCG that had at least one aCL. This finding indicates that an important strategy in future studies should be to improve hCG responses by inducing a greater aCL development. The effectiveness of the hormonal treatment regimen used in the current study during the non-breeding season was evidenced in the greater percentage of lambs born per number of ewes responding to the estrous synchrony treatment regimen in G-hCG group. Furthermore, lamb weight and litter weight at birth were similar between treatment groups, indicating that lamb uterine development was not affected by the treatment regimens imposed. The present report is probably the first in which there was a focus on the use of hCG to induce aCL in farm animals.

Table 4
Indices of reproductive performance of Morada Nova ewes that on Day 7.5, after imposing a synchronous estrous induction treatment regimen that were treated with either 300 IU hCG (G-hCG) or 1 mL of saline solution (G-Control) during the non-breeding season.

Variables	G-Control	G-hCG	*P*-value
Rate of ewes with aCL formed (%)	0.0 (0/53)	81.5 (44/55)	<.0001
Pregnancy rate (%)	47.1 (25/53)	60.0 (33/55)	0.25
Parturition rate (%)	92.0 (23/25)	96.9 (32/33)	1.00
Total pregnancy loss (%)	8.0 (2/25)	6.1 (2/33)	1.00
Multiple birth rate (%)	56.5 (13/23)	46.8 (15/32)	0.66
Total number of lambs born	35	50	–
Prolificacy	1.5 ± 0.5	1.5 ± 0.7	0.98
Rate of number of lambs born/number of synchronized ewes (%)	66.0 (35/53)	90.9 (50/55)	0.003
Lamb weight at birth (kg)	2.7 ± 0.5	2.6 ± 0.7	0.21
Litter weight at birth (kg)	3.9 ± 1.3	3.8 ± 1.4	0.62

* () number of animals.

5. Conclusions

There was administration to treatment group of Morada Nova ewes in the present study of hCG 7.5 days after removal from the vagina of a progestin-containing sponge during the non-breeding season. There was induction of aCL development, in the hCG-treated group with there being greater values for variables related to the luteal tissue functions and P4 concentrations in ewes of this group compared with the ewes of the control group. Follicles that were 3.5 mm in diameter could be induced with the hCG treatment to develop into aCL. Although the overall pregnancy rate was not different between ewes of the hCG treated and control group, ewes with aCL development had a greater pregnancy rate. The hormonal regimen imposed in the present study resulted in an enhanced value for the most important variables for sheep production, the number of lambs born per ewes placed with rams during the breeding season.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

Acknowledgments

We thank all staff from Embrapa for help and support for this experiment. The authors also thank Embrapa (Project 22.13.06.026.00.06) for financial aid. GBV, MEFO, JFF and JMGS-F are CNPq fellows and JMGS-F is a FAPERJ fellow and JFF a FAPEMIG fellow.

References

Allam, A.M., El-Zarkouny, S.Z., Elshahawy, I.I., Nagadi, S.A., Kesler, D.J., 2015. Effects of post-mating treatments with gonadotropin-releasing hormone and equine chorionic gonadotropin on reproductive efficiency of Harri ewes in tropical regions. Small Rumin. Res. 132, 55–59. https://doi.org/10.1016/j.smallrumres.2015.10.002.

Arashiro, E.K.N., Ungerfeld, R., Clariget, R.P., Pinto, P.H.N., Balaro, M.F.A., Bragança, G.M., Ribeiro, L.S., Fonseca, J.F., Brandão, F.Z., 2018. Early pregnancy diagnosis in ewes by subjective assessment of luteal vascularisation using colour Doppler ultrasonography. Theriogenology 106, 247–252. https://doi.org/10.1016/j.theriogenology.2017.10.029.

Binelli, M., Thatcher, W.W., Mattos, R., Baruselli, P.S., 2001. Antiluteolytic strategies to improve fertility in cattle. Theriogenology 56 (9), 1451–1463. https://doi.org/10.1016/S0093-691X(01)00646-X.

Box, G.E.P., Cox, D.R., 1964. An analysis of transformations. J. R. Stat. Soc. Ser. B 26, 211–243.

Carvalho-de-Paula, C.J., Souza-Fabjan, J.M.G., Gonçalves, J.D., Dias, J.H., de Souza, G.N., Oliveira, M.E.F., Fonseca, J.F., 2020. Effect of a 12-h increment in the short-term treatment regimen on ovarian status, estrus synchrony, and pregnancy rate in artificially inseminated dairy goats. Anim. Reprod. Sci. 106571. https://doi.org/10.1016/j.anireprosci.2020.106571.

Catalano, R., Teruel, M., González, C., Williams, S., Dorna IV, Callejas, S., 2015. Reproductive performance of ewe lambs in non-breeding season exposed to hCG at day 12 post mating. Small Rumin. Res. 124, 63–67. https://doi.org/10.1016/j.smallrumres.2014.12.014.

Cavalcanti, A.S., Brandão, F.Z., Garcia Nogueira, L.A., Fonseca, J.F., 2012. Effects of GnRH administration on ovulation and fertility in ewes subjected to estrous synchronization. R. Bras. Zootec. 41, 1412–1418. https://doi.org/10.1590/S1516-35982012000600014.

Coleson, M.P., Sanchez, N.S., Ashley, A.K., Ross, T.T., Ashley, R.L., 2015. Human chorionic gonadotropin increases serum progesterone, number of corpora lutea and angiogenic factors in pregnant sheep. Reproduction 150 (1), 43–52. https://doi.org/10.1530/REP-14-0632.

Cosentino, I.O., Balaro, M.F.A., Leal, F.S.C., da Silva Carvalho, A.B., de Souza, P.R.C., Arashiro, E.K.N., Brandão, F.Z., 2018. Accuracy of assessment of luteal morphology and luteal blood flow for prediction of early pregnancy in goats. Theriogenology 121, 104–111. https://doi.org/10.1016/j.theriogenology.2018.08.007.

Driancourt, M.A., 2001. Regulation of ovarian follicular dynamics in farm animals. Implications for manipulation of reproduction. Theriogenology 55 (6), 1211–1239. https://doi.org/10.1016/S0093-691X(01)00479-4.

Evans, A.C.O., Duffy, P., Hynes, N., Boland, M.P., 2000. Waves of follicle development during the estrous cycle in sheep. Theriogenology 53 (3), 699–715. https://doi.org/10.1016/S0093-691X(99)00268-X.

Fernandez, J., Bruno-Galarraga, M.M., Soto, A.T., de la Sota, R.L., Cueto, M.I., Lacau, I.M., Gibbons, A.E., 2018. Hormonal therapeutic strategy on the induction of accessory corpora lutea in relation to follicle size and on the increase of progesterone in sheep. Theriogenology 105, 184–188. https://doi.org/10.1016/j.theriogenology.2017.09.020.

Fernandez, J., Bruno-Galarraga, M.M., Soto, A.T., de la Sota, R.L., Cueto, M.I., Lacau-Mengido, I.M., Gibbons, A.E., 2019. Effect of GnRH or hCG administration on Day 4 post insemination on reproductive performance in Merino sheep of North Patagonia. Theriogenology 126, 63–67. https://doi.org/10.1016/j.theriogenology.2018.12.008.

Figueira, L.M., Fonseca, J.F., Arashiro, E.K.N., Souza-Fabjan, J.M.G., Ribeiro, A.C.S., Oba, E., Viana, J.H.M., Brandão, F.Z., 2015. Colour Doppler ultrasonography as a tool to assess luteal function in Santa Inês ewes. Reprod. Domest. Anim. 50 (4), 643–650. https://doi.org/10.1111/rda.12543.

Fonseca, J.F., Torres, C.A.A., 2005. Administration of hCG 5 days after breeding and reproductive performance in nulliparous dairy goats. Reprod. Domest. Anim. 40 (6), 495–499. https://doi.org/10.1111/j.1439-0531.2005.00612.x.

Fonseca, J.F., Filho, J.M.S., Neto, A.P., Palhares, M.S., Ruas, J.R.M., Alvim, M.T.T., Belissário, H., Pardini, W.S., 2000. Indução de corpo lúteo acessório em novilhas (induction of accessory corpus luteum in heifers). Rev. Bras. Reprod. Anim. 24 (3), 143–146.

Fonseca, J.F., Silva Filho, J.M., Palhares, M.S., Ruas, J.R.M., Pinto Neto, A., 2001. Concentração plasmática de progesterona em novilhas receptoras submetidas à administração de rbST, GnRH ou hCG no quinto dia do ciclo estral. Arq. Bras. Med. Vet. Zootec. 53 (4), 1–8. https://doi.org/10.1590/S0102-09352001000400012.

Fonseca, J.F., Maffili, V.V., Rodrigues, M.T., Santos, A.D.F., Rovay, H., Pinto Neto, A., Brandão, F.Z., Tores, C.A.A., 2006. Effects of hCG on progesterone concentrations and fertility in cyclic, lactating Alpine goats. Anim. Reprod. 3 (4), 410–414.

Fonseca, J.F., Castro, A.C.R., Arashiro, E.K.N., Oliveira, M.E.F., Zambrini, F.N., Esteves, L.V., Brandão, F.Z., Souza-Fabjan, J.M.G., 2018. Effects of hCG administration on accessory corpus luteum formation and progesterone production in estrous-induced nulliparous Santa Inês ewes. Anim. Reprod. 15 (2), 135–139. https://doi.org/10.21451/1984-3143-AR2017-957.

Ford, S.P., 1985. Maternal recognition of pregnancy in the ewe, cow and sow: vascular and immunological aspects. Theriogenology 23 (1), 145–159.

Forde, N., Lonergan, P., 2017. Interferon-tau and fertility in ruminants. Reproduction 154 (5), 33–43. https://doi.org/10.1530/REP-17-0432.

Khan, T.H., Beck, N.F., Khalid, M., 2007. The effects of GnRH analogue (buserelin) or hCG (Chorulon) on Day 12 of pregnancy on ovarian function, plasma hormone concentrations, conceptus growth and placentation in ewes and ewe lambs. Anim. Reprod. Sci. 102 (3-4), 247–257. https://doi.org/10.1016/j.anireprosci.2006.11.007.

Khan, T.H., Beck, N.F.G., Khalid, M., 2009. The effect of hCG treatment on Day 12 post-mating on ovarian function and reproductive performance of ewes and ewe lambs. Anim. Reprod. Sci. 116 (1-2), 162–168. https://doi.org/10.1016/j.anireprosci.2009.01.010.

Lankford, L., Yates, D., Halalsheh, R., Black, P., Hallford, D., Ross, T., 2010. Effects of human chorionic gonadotropin on serum progesterone concentrations, embryonic survival, and lambing rates in ewes. Western Section, Am. Soc. Anim. Sci. 61, 153-15. https://eis.hu.edu.jo/deanshipfiles/pub111606023.pdf (Accessed 20 April 2020).

Lonergan, P., Forde, N., Spencer, T., 2016. Role of progesterone in embryo development in cattle. Reprod. Fert. Develop. 28 (2), 66–74. https://doi.org/10.1071/RD15326.

Maillo, V., Duffy, P., O'Hara, L., De Frutos, C., Kelly, A.K., Lonergan, P., Rizos, D., 2014. Effect of hCG administration during corpus luteum establishment on subsequent corpus luteum development and circulating progesterone concentrations in beef heifers. Reprod. Fert. Develop. 26 (3), 367–374. https://doi.org/10.1071/RD12353.

Menchaca, A., Rubianes, E., 2004. New treatments associated with timed artificial insemination in small ruminants. Reprod. Fert. Develop. 16 (4), 403–413. https://doi.org/10.1071/RD04037.

Oliveira, M.E.F., Feliciano, M.A.R., D'Amato, C.C., Oliveira, L.G., Bicudo, S.D., Fonseca, J.F., Vicente, W.R.R., Visco, E., Bartlewski, P.M., 2014. Correlations between ovarian follicular blood flow and superovulatory responses in ewes. Anim. Reprod. Sci. 144, 30–37. https://doi.org/10.1016/j.anireprosci.2013.10.012.

Oliveira, M.E.F., Ayres, H., Oliveira, L.G.D., Barros, F.F.P.D.C., Oba, E., Bicudo, S.D., Bartlewski, P.M., Fonseca, J.F., Vicente, W.R.R., 2016. Effects of season and ovarian status on the outcome of long-term progesterone-based estrus synchronization protocols and ovulatory follicle development in Santa Ines ewes under subtropical conditions. Theriogenology 85 (3), 452–460. https://doi.org/10.1016/j.theriogenology.2015.09.024.

Oliveira, M.E.F., Bartlewski, P.M., Jankowski, N., Padilha-Nakaghi, L.C., Oliveira, L.G., Bicudo, S.D., Fonseca, J.F., Vicente, W.R.R., 2017. Relationship of antral follicular blood flow velocity to superovulatory responses in ewes. Anim. Reprod. Sci. 182, 48–55. https://doi.org/10.1016/j.anireprosci.2017.04.009.

Oliveira, M.E.F., Ribeiro, I.F., Rodriguez, M.G.K., Maciel, G.S., Fonseca, J.F., Brandão, F.Z., Bartlewski, P.M., 2018. Assessing the usefulness of B-mode and colour Doppler sonography, and measurements of circulating progesterone concentrations for determining ovarian responses in superovulated ewes. Reprod. Domest. Anim. 53 (3), 742–750. https://doi.org/10.1111/rda.13165.

Pugliesi, G., Miagawa, B.T., Paiva, Y.N., França, M.R., Silva, L.A., Binelli, M., 2014. Conceptus-induced changes in the gene expression of blood immune cells and the ultrasound-accessed luteal function in beef cattle: how early can we detect pregnancy? Biol. Reprod. 91 (4) https://doi.org/10.1095/biolreprod.114.121525, 95–1.

Rhind, S.M., Chesworth, J.M., Robinson, J.J., 1978. A seasonal difference in ovine peripheral plasma prolactin and progesterone concentrations in early pregnancy and in the relationship between the two hormones. Reproduction 52, 79–81. https://doi.org/10.1530/jrf.0.0520079.

Satterfield, M.C., Bazer, F.W., Spencer, T.E., 2006. Progesterone regulation of preimplantation conceptus growth and galectin 15 (LGALS15) in the ovine uterus. Biol. Reprod. 75 (2), 289–296. https://doi.org/10.1095/biolreprod.106.052944.

Schmitt, E.P., Barros, C.M., Fields, P.A., Fields, M.J., Diaz, T., Kluge, J.M., Thatcher, W.W., 1996. A cellular and endocrine characterization of the original and induced corpus luteum after administration of a gonadotropin-releasing hormone agonist or human chorionic gonadotropin on day five of the estrous cycle. Anim. Sci. J. 74 (8), 1915–1929. https://doi.org/10.2527/1996.7481915x.

Teixeira, T.A., Fonseca, J.F., Souza-Fabjan, J.M.G., Carvalheira, L.R., Fernandes, D.A.M., Brandão, F.Z., 2016. Efficiency of different hormonal treatments for estrus synchronization in tropical Santa Inês sheep. Trop. Anim. Health Pro. 48, 545–551. https://doi.org/10.1007/s11250-015-0989-y.

Veenhuizen, E.L., Wagner, J.F., Tonkinson, L.V., 1972. Corpus luteum response to 6-chloro Δ6-17 acetoxyprogesterone and hCG in the cow. Biol. Reprod. 6 (2), 270–276. https://doi.org/10.1093/biolreprod/6.2.270.

Villaquiran, M., Gipson, T., Merkel, R.C., Goetsch, A., Sahlu, T., 2007. Body Condition Scores in Goats (Accessed 01 September 2020). 22. 125-131. Ann. Goat Field Day, Langston University, Langston, OK. https://www.researchgate.net/publication/264889567_Body_Condition_Scores_in_Goats.

Viñoles, C., Meikle, A., Forsberg, M., Rubianes, E., 1999. The effect of subluteal levels of exogenous progesterone on follicular dynamics and endocrine patterns during the early luteal phase of the ewe. Theriogenology 51 (7), 1351–1361. https://doi.org/10.1016/S0093-691X(99)00079-5.

Yen, S.S.C., Llerena, O., Little, B., Pearson, O.H., 1968. Disappearance rates of endogenous luteinizing hormone and chorionic gonadotropin in man. J. Clin. Endocrinol. Metab. 28 (12), 1763–1767. https://doi.org/10.1210/jcem-28-12-1763.