



Hormonal protocols for early resynchronization of ovulation in ewes: The use of progestagens, eCG, and inclusion of early pregnancy diagnosis with color Doppler ultrasound

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ABSTRACT

This study aimed to evaluate 1) the effect of inserting an intravaginal sponge containing medroxyprogesterone during the late luteal phase on the corpus luteum (CL) function and endogenous production of P4; 2) the effectiveness of two different equine chorionic gonadotrophin (eCG) doses on synchronization of ovulations for a resynchronization treatment; and 3) the inclusion of an early pregnancy diagnosis in an early resynchronization protocol for ovulation in ewes. For all studies, the synchronization protocol was based on a short-term protocol of six days of progestogen treatment plus one dose of prostaglandin F2alpha, one dose of eCG, and gonadorelin acetate after sponge withdrawal. For the first study, the ewes were mated with fertile rams; a second sponge was inserted in half of these ewes 12 days later, and blood samples were collected daily for six days, until sponge withdrawal. For the second study, the ewes were not mated, and received a second sponge during the same period, after which they were divided into three groups according to eCG dose (0, 200, or 300 IU). In the third study, all ewes were artificially inseminated and received the second sponge during the same period. At sponge withdrawal, pregnancy was diagnosed by color Doppler ultrasonography (DUS) of the CL, and only non-pregnant ewes were re-inseminated two days later. In the first study, serum progesterone values were similar regardless of whether an intravaginal sponge had been inserted. In the second study, the ovulation time was more concentrated in those ewes which received 200 IU of eCG. In the third study, there was no difference between the experimental groups (with or without a previous pregnancy diagnosis) in pregnancy rate at the first insemination, accumulated pregnancy rate, and pregnancy loss. The insertion of an intravaginal sponge impregnated with medroxyprogesterone acetate did not affect the endogenous production of P4. The application of 200 IU of eCG provided the best result with regard to the synchronization of ovulations in the resynchronization treatment. Also, the inclusion of an early pregnancy diagnosis with DUS is useful and improves the general results of resynchronization programs, shortening the total working period.

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1. Introduction

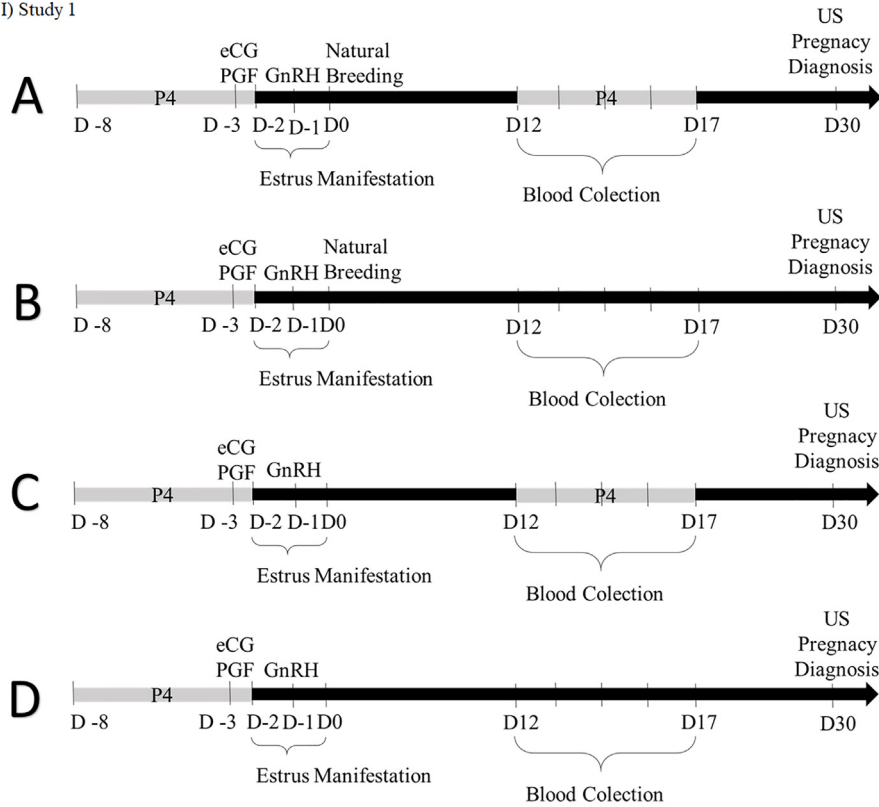
An early pregnancy diagnosis increases reproductive efficiency, especially in that non-pregnant females can be identified and re-inseminated as early as possible, thus reducing inter-insemination and lambing intervals [1]. In this sense, pregnancy can be accurately diagnosed as soon as 17 days after mating in ewes

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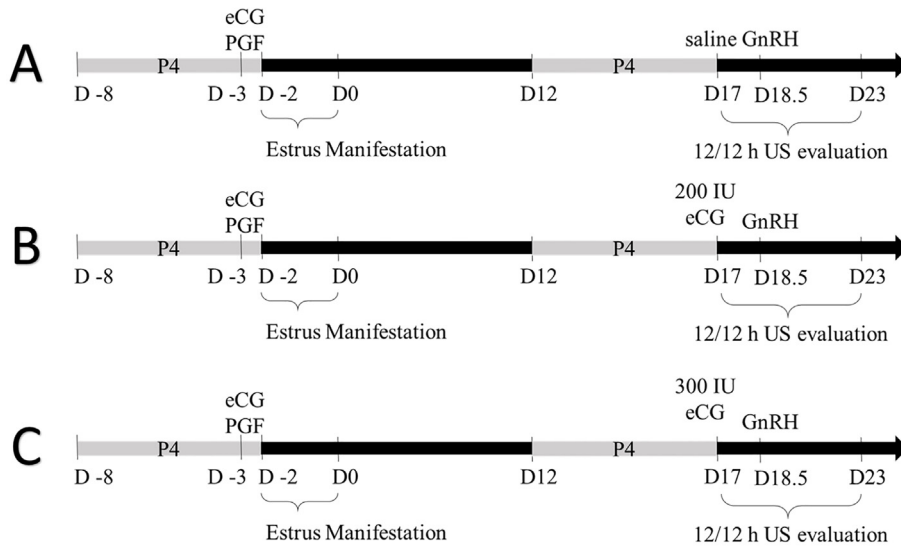
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I) Study 1



II) Study 2



III) Study 3

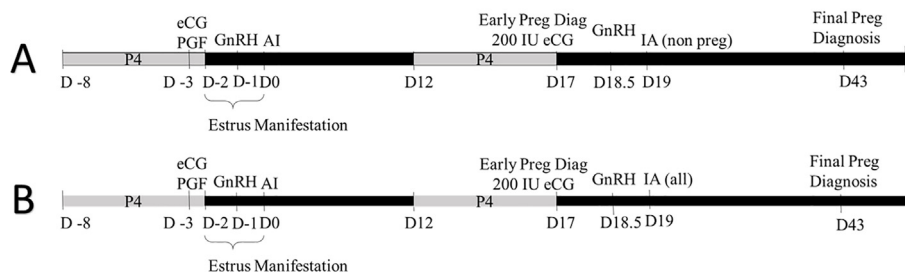


Fig. 1. (I) Study 1 methodology: A) G_{PSP} : pregnant animals with sponge; B) G_{PNSP} : pregnant animals without sponge; C) G_{NPSp} : non-pregnant animals with sponge; and D) G_{NPNSP} : non-pregnant animals without sponge. (II) Study 2 methodology: A) $Group_{CON}$; B) $Group_{eCG200}$; C) $Group_{eCG300}$. (III) Study 1 methodology: A) $Group_{EPD}$; B) $Group_{NEP}$.

by assessing the luteal blood flow using color Doppler ultrasonography (DUS). This technique may be included in treatments for estrous resynchronization, reducing the time required between synchronization and re-synchronization of ovulations [2–5]. Hormonal protocols for resynchronizing ovulation have been applied to all previously inseminated ewes without knowing which were pregnant and which were not [3]. However, administering these treatments to the whole flock is costly, time-consuming, and laborious, and increases the use of unnecessary hormones as many treated ewes are pregnant from the previous insemination. Moreover, pregnant ewes are not unnecessarily moved for the re-insemination treatment to be administered, thus avoiding any decrease in grazing time. Therefore, if an early pregnancy diagnosis with DUS is included, treatments can be continued in non-pregnant ewes only to improve artificial insemination programs.

In cattle, treatments to resynchronize the ovulation after an initial Fixed-Time Artificial Insemination (FTAI) or natural breeding have already been used successfully [2,5,6]. These hormonal protocols require a combination of different drugs (P4, GnRH analogue) differing in dose and/or time of administration [2,4,6]. However, it is not known if the application of such hormonal treatments, especially an exogenous source of P4, could affect the functionality and maintenance of the corpus luteum (CL) in early-pregnant ewes. In cows, an exogenous source of P4 administered through intravaginal devices before pregnancy diagnosis did not produce luteolysis, and thus did not affect the conception rate of resynchronization treatments [4]. This could have happened due to the negative feedback of external P4 on the functionality and viability of the CL, which did not occur in that study [4].

Equine chorionic gonadotropin (eCG) is widely used in hormonal protocols to induce the final follicular growth and ovulation [7,8]. In FTAI protocols, eCG may be included to synchronize ovulations. In this sense, greater doses of eCG associated with progesterone devices increase pregnancy rates in synchronization protocols [9,10]; however, little is known about the relationship between eCG dose and the synchronization of ovulations in a second eCG application administered for this purpose.

Therefore, this study aimed to determine in ewes: (1) whether the insertion of an intravaginal sponge containing medroxyprogesterone during the late luteal phase affects the functionality of the CL and the endogenous production of P4; (2) the effectiveness of two different eCG doses on synchronization of ovulations for a resynchronization treatment; and (3) whether an early pregnancy diagnosis should be included in the protocol for early resynchronization of ovulations.

2. Materials and methods

The procedures of Studies 1 and 2 were approved by the Comissão de Ética no Uso de Animais of the Universidade Federal Fluminense (Brazil) (protocol #923/2017). These trials were carried out according to the ethical principles of the Colégio Brasileiro de Experimentação Animal (COBEA). The animal management and experimental procedures of the third study were approved by the Comité de Ética en el Uso de Animales de Experimentación of the Facultad de Agronomía, Universidad de la República (Uruguay) (protocol 021130-000373-18).

2.1. Study 1

The study was conducted at Unidade de Pesquisa Experimental em Caprinos e Ovinos of the Universidade Federal Fluminense, located in Cachoeiras de Macacu (22°S, 42°W), Rio de Janeiro, Brazil (tropical hot-humid climate type – Aw [11]). A total of 42 Santa Ines x Dorper adult ewes with a body condition score of 3.0 ± 0.3

(mean \pm SD) (scale 1–5 [12]) were used. All ewes were subjected to a gynecological examination before the study to ensure that none had reproductive abnormalities detectable by ultrasound or clinical examination. Throughout the study, ewes were kept in a semi-intensive system under grazing supplemented with chopped grass (*Pennisetum purpureum*) at 2.5 kg DM/kg of their live weight. Also, a concentrate at 0.5 kg DM/kg of their live weight (12% of crude protein) was provided as needed [13]. Water and mineral salt were provided *ad libitum*.

2.1.1. Estrous synchronization treatments

All ewes were treated with an estrous synchronization protocol adapted from Balaro et al. [7] (Fig. 1I). Briefly, a sponge impregnated with 60 mg of medroxyprogesterone acetate (Progespon, Schering Plough, São Paulo, Brazil) was inserted in each animal, and remained *in situ* for six days. Twenty-four hours before sponge withdrawal, all animals received 0.24 mg of cloprostenol sodium (Estron, Agner União, São Paulo, Brazil) and 300 IU of eCG (Novormon, Schering Plough, São Paulo, Brazil). All ewes also received 0.025 mg of gonadorelin acetate (GnRH – Gestran Plus, Tecnopec, São Paulo, Brazil) 36 h after sponge withdrawal. To ensure a similar number of pregnant and non-pregnant animals, only 24 ewes were mated with fertile rams. The estrous behavior of those ewes was checked twice a day, and they were mated once while receptive.

A second intravaginal sponge was inserted on Day 12 (Day 0 = 56 h after sponge withdrawal) in 12 animals submitted for natural breeding and in nine of those that were not bred. Sponges remained *in situ* until Day 17, when the early pregnancy diagnosis was performed with DUS according to Arashiro et al. [14]. Briefly, CL blood perfusion was assessed and classified in scores 1–4, where 1 corresponded to non-pregnant ewes, and 2 to 4 to pregnant ewes. The ultrasound scan was performed with a portable device (Sonoscape S6, Shenzhen, China) coupled to a 7.5 MHz linear rectal transducer adapted for use with small ruminants. The Doppler settings used in the luteal assessments follow in sequence: 20% color gain, pulse repetition frequency (PRF) of 1.0 kHz, 7 cm of depth, and wall filter (WF) of 75 MHz.

After a final pregnancy diagnosis on Day 30 by uterus scan using B-Mode ultrasound (gold-standard method), the animals were retrospectively categorized in groups as follows: G_{PSP} : pregnant animals with sponge ($n = 10$); G_{PNSP} : pregnant animals without sponge ($n = 9$); G_{NPNSP} : non-pregnant animals with sponge ($n = 11$), and G_{NNPNSP} : non-pregnant animals without sponge ($n = 12$). In non-pregnant animals, the onset of luteolysis was considered to be the moment when plasma progesterone concentration decreased more than 50% when compared to the previous day, followed by a decrease to values lower than 1 ng/mL on the subsequent day.

2.1.2. Blood sample collection and serum P4 concentration

Blood samples were collected daily from Day 12 to Day 17 by jugular venipuncture, using tubes (without anti-coagulant) with a vacuum system. Blood samples were centrifuged at $1000 \times g$ for 15 min; serum was separated, and stored at -20°C until analysis. Serum P4 concentration was determined by radioimmunoassay using commercial kits (MP Biomedicals, LLC, Diagnostics Division, Orangeburg, NY, USA). Sensitivity and intra-assay coefficient of variation were 0.05 ng/mL and 12%, respectively. All data were within minimum and maximum points of the curve.

2.2. Study 2

The second study (Fig. 1II) was conducted at the same experimental farm, and under the same nutritional conditions, using 30 adult Santa Ines x Dorper ewes (body weight: 47.8 ± 5.6 kg; body

condition score: 2.9 ± 0.3).

2.2.1. Estrous synchronization, resynchronization treatments, and ultrasound assessment

All ewes were treated with the same estrous synchronization treatment used in the first study. However, ewes were not mated with the ram. Then, a second intravaginal sponge impregnated with 60 mg of medroxyprogesterone was inserted on Day 12 (Day 0 = 56 h after sponge withdrawal) and maintained *in situ* until Day 17 in all the animals. On Day 17, ewes were randomly allocated to three experimental groups ($n = 10$ each) according to the eCG (Novormon, Schering Plough, São Paulo, Brazil) dose administered i.m. simultaneously with the sponge withdrawal. Ewes from Group_{Con} received 1.0 mL of saline solution, and those from Group_{eCG200} and Group_{eCG300} received 200 IU and 300 IU of eCG, respectively. Thirty-six hours after sponge withdrawal, all ewes received 0.025 mg of gonadorelin acetate (Gestran Plus, Tecnopec, São Paulo, Brazil).

The ovaries of all ewes were observed by ultrasound scan every 12 h from the second sponge withdrawal until ovulation (confirmed by the disappearance of a previous dominant follicle(s) greater than 5 mm). Estrous behavior was also determined every 12 h by fertile rams.

2.3. Study 3

The third study was conducted at Estación Experimental Bernardo Rosengurt of Facultad de Agronomía, Universidad de la República, located in Cerro Largo (32°S, 54°W), Uruguay (humid subtropical climate type – Cfa [11]). A total of 188 adult Corriedale ewes (body weight: 49.2 ± 6.2 kg; body condition score: 3.27 ± 0.03) were used during the breeding season (April). Animals grazed under extensive conditions on natural grassland at approximately 3.0 kg DM/kg of their live weight, with free access to water.

2.3.1. Estrous synchronization and artificial insemination

All ewes were treated with the same protocol used in the first and second studies with the exception of the GnRH analogue (gonadorelin, Gonasyn, Zoetis, Montevideo, Uruguay), which was administered 24 h after sponge withdrawal (0.050 mg i.m.) (Fig. 1.III). Estrous behavior was recorded twice daily with vasectomized rams, and ewes were inseminated 27–30 h after GnRH administration with fresh semen collected from two fertile Corriedale rams. Semen doses, which had approximately 2×10^8 spermatozoa in 0.2 mL, determined by a photometer, were inserted using a speculum equipped with a light source and a multidose insemination gun (Walmur Veterinary Instrument, Montevideo, Uruguay), which provided a superficial cervical insemination.

2.3.2. Ultrasonographic procedures and resynchronization of ovulation

A second intravaginal sponge (Progespon, Syntex, Buenos Aires, Argentina) was inserted on Day 12 (Day 0 = 51–54 h after sponge withdrawal) and maintained *in situ* until Day 17 in all animals. Pregnancy was determined on Day 17 according to Arashiro et al. [14] using a portable DUS device (Esaote, MyLabOne, Genoa, Italy) equipped with a 7.5 MHz linear rectal transducer. The Doppler settings used in the luteal assessments follow in sequence: 32% color gain, PRF of 2.0 kHz, 7 cm of depth, and WF of 90 MHz.

The females were randomly divided in two groups: 1) Group_{EPD}: the early pregnancy diagnosis was performed, and therefore, only non-pregnant ewes were resynchronized; and 2) Group_{NEP}: all ewes were treated regardless of gestational status.

All sponges were removed on Day 17, and non-pregnant ewes

from Group_{EPD} and all ewes from Group_{NEP} received 200 UI of eCG i.m. (Novormon, Zoetis, Montevideo, Uruguay) according to the results of the second study. The same ewes received 0.050 mg of gonadorelin 36 h after sponge withdrawal, and then underwent a second insemination 9–12 h later (Day 19). Forty days after the second insemination, pregnancy diagnosis was performed by transrectal ultrasound.

After the final pregnancy diagnosis was performed, the results of the first and second AI were compared in each experimental group. In sequence, the accumulated pregnancy (ewes diagnosed as pregnant at the first and second), accumulated non-pregnant (ewes that were not pregnant at both moments), and pregnancy losses (ewes that were diagnosed as pregnant at the first but non-pregnant at the second) were calculated.

2.4. Data analysis

The Lilliefors test was used to verify data normality. Data were analyzed with SAS (University Edition version). In the first study, progesterone values were analyzed separately for the ewes that were or were not pregnant using a mixed model procedure including the group (treated or not treated), time, and their interaction as main effects in the model. LS means were compared with the pdiff option of the mixed model. In the second study, dispersion of the data (homoscedasticity) was compared by using Bartlett's test. The time from sponge withdrawal to ovulation, and that from estrus onset to ovulation, were compared with a mixed model including the treatments as main factors. The frequency of ewes with single or multiple ovulations was compared with Fisher's exact probability test. In the third study, pregnancy rate, accumulated pregnancy rate, and possible pregnancy losses were compared among groups using the chi-square test. Differences were considered as statistically significant when $P \leq 0.05$.

3. Results

3.1. Study 1

From Day 12 to Day 17, serum progesterone values did not differ between pregnant ewes with or without the second sponge (G_{PSP} and G_{PNSP}, respectively; Fig. 2A). In non-pregnant animals, the luteolysis time did not differ between groups (Days 14.6 ± 1.1 and 14.6 ± 0.9 for G_{NPNP} and G_{NPSB}, respectively), and the serum progesterone did not differ before luteolysis in non-pregnant ewes (Fig. 2B).

3.2. Study 2

There were no differences among groups (G_{Con}, G_{eCG200}, and G_{eCG300}) in the time from sponge withdrawal to ovulation, or time from estrus onset to ovulation (Table 1). However, ovulation time was more concentrated in G_{eCG200} than in G_{Con} and G_{eCG300} treated ewes. The frequency of ewes with single or double ovulations did not differ among groups (8/10, 8/9, and 6/7 ewes with single ovulations from G_{Con}, G_{eCG200}, and G_{eCG300}, respectively).

3.3. Study 3

There was no difference between experimental groups (with or without previous pregnancy diagnosis) in pregnancy rate at the first insemination, accumulated pregnancy rate, and pregnancy losses (Table 2).

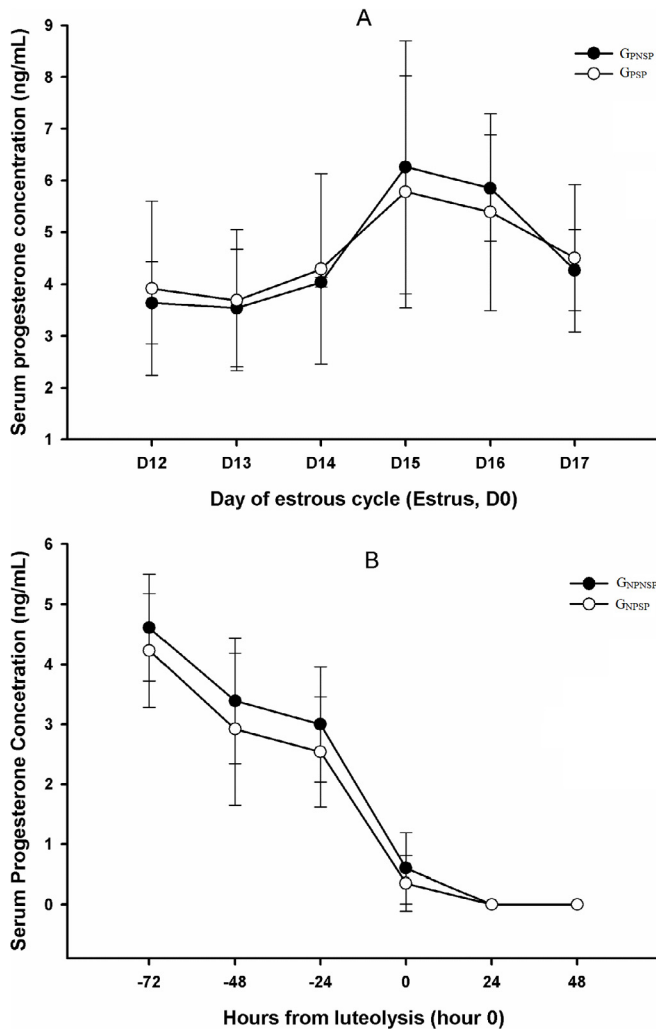


Fig. 2. Serum progesterone concentration (ng/mL) from Day 12 to Day 17, in pregnant (A) and non-pregnant (B) ewes. An intravaginal sponge impregnated with medroxyprogesterone acetate was inserted in ewes from the G_{NPSP} and G_{PSP} groups.

Table 1

Time from sponge withdrawal to ovulation, and from estrous onset to ovulation, in ewes that received 0 (group Con), 200 (group eCG200), or 300 (group eCG300) IU of eCG during a treatment for resynchronization of ovulations (LSmean ± SEM).

	Con	eCG200	eCG 300
Sponge withdrawal to ovulation (h)	46.8 ± 11.5 ^a	56.2 ± 3.8 ^b	54.1 ± 14.9 ^a
Estrous onset to ovulation (h)	19.2 ± 14.7	25.8 ± 10.0	20.9 ± 14.1

^{a,b} means a significant difference in dispersion among treatments.

Table 2

Pregnancy rate and accumulated pregnancy in ewes subjected to treatments for synchronization and resynchronization of ovulation with or without pregnancy diagnosis with color DUS 17 days after first insemination.

	Pregnancy at D17			Non-pregnancy at D17		Accumulated pregnancy at D59	Accumulated non-pregnancy at D59
	Pregnancy at D17	Pregnancy at D59	Non-pregnancy at D59	Pregnancy at D59	Non-pregnancy at D59		
Group _{EPD}	21.7% (20/92)	75.0% (15/20)	25.0% (5/20)	45.8% (33/72)	54.1% (39/72)	52.1% (48/92)	47.9% (44/92)
Group _{NEP}	19.1% (18/94)	66.7% (12/18)	33.3% (6/18)	38.1% (29/76)	61.8% (47/76)	43.6% (41/94)	56.4% (53/94)
Total	19.4% (38/186)	71.1% (27/38)	28.9% (11/38)	41.9% (62/148)	58.1% (86/148)	47.8% (89/186)	52.2% (97/186)

Group_{EPD}: the early pregnancy diagnosis was performed and, therefore, only non-pregnant ewes continued with the treatment for resynchronization of ovulation; Group_{NEP}: all ewes continued with the treatment regardless of gestational status.

Accumulated pregnancy (females that were diagnosed as pregnant at the first and second pregnancy diagnosis), accumulated non-pregnant (ewes that were not pregnant at both moments), and pregnancy losses (females that were diagnosed as pregnant at the first but non-pregnant at the second pregnancy diagnosis).

4. Discussion

To the best of our knowledge, these were the first studies directed at the implementation of protocols of resynchronization of ovulations for FTAI in ewes including early pregnancy diagnosis with DUS. In general, our results indicate that, the second sponge did not negatively influence early pregnancy, and it was possible to determine that 200 IU was the most effective dose of eCG to synchronize the ovulation. The use of DUS was useful in determining which ewes should undergo a second hormone protocol and FTAI, and thus simplify treatments and avoid the unnecessary movements of animals, with practical implications for simplifying the use of these techniques, decreasing cost, increasing the grazing time of pregnant ewes, and preventing them from being subjected to stressors during this key period.

Our results demonstrated clearly that the use of a second sponge does not interfere with the luteolysis or pregnancy from the first FTAI. Although Miranda et al. [3] proposed a negative effect of medroxyprogesterone devices on progesterone production, in that study there were no negative effects on the final percentage of pregnant ewes. In line with this, in our study progesterone concentration patterns were similar in ewes that received or did not receive a second sponge. Therefore, although the use of a sponge in those ewes in which pregnancy had not yet been detected implies the inclusion of an unnecessary step for the final result, it does not have negative effects on the ovarian activity of the ewes, and thus allows the treatment to be applied.

The administration of 200 IU was the most effective dose for synchronizing the ewes' ovulation. Greater doses of eCG may be more effective for induction of estrus and ovulation in non-cycling ewes [9,10], but there is scarce information on the effects of the dose on the dispersion of the ovulations. A mild eCG dose such as 200 IU may produce less interference in the endogenous LH peak and, subsequently, obtain a more homogenous hormonal response and ovulation in ewes. In this sense, greater doses of eCG, such as those used for superovulatory treatments, advance the release of the endogenous LH peak, and may cause greater dispersion of ovulation among different animals [15,16]. It is also important to highlight that the ewes had their ovarian cycle pre-synchronized due to the first protocol, and therefore a low dose might have been enough to obtain a good synchronization in the second protocol.

The inclusion of the early pregnancy diagnosis did not modify the final pregnancy rate. Doppler examination is increasingly being used in livestock for ovarian assessment for early pregnancy diagnosis in cows [17], ewes [14], and does [18]. This is a very important result as, at the same time, the treatments ended for all the pregnant ewes, thus decreased the management of the animals, the costs, the loss of an important number of semen doses, and the use of unnecessary hormones. Moreover, ewes were not unnecessarily

moved, allowing them to graze longer and preventing stressors, thus improving their welfare. This decrease of hormones has a further practical impact on ewes' welfare, as no pregnant animal received more hormonal treatments or was subjected to management for artificial insemination while pregnant. Changing the moment at which GnRH was administered in the first FTAI of this study meant the number of pregnant ewes was low compared to that traditionally obtained. This explains the low number of ewes that were not pregnant at the second ultrasound although they had been considered pregnant at the first. That slight difference can be due to early pregnancy losses, which can occur in ewes until the 65th day [19], or to errors in the interpretation of the Doppler images, as there may be 8.6–14.3% of false positives when the early pregnancy diagnosis is carried out 17 days after mating [14]. Overall, it is important to highlight that the inclusion of the early pregnancy diagnosis does not undermine the results of the first FTAI, with several practical advantages.

5. Conclusions

The insertion of an intravaginal sponge impregnated with medroxyprogesterone acetate did not affect the endogenous production of P4. The application of 200 IU of eCG proved the best means to synchronize the ovulation of the resynchronization treatment, leading to a better determination of the time for FTAI. Also, the inclusion of an early pregnancy diagnosis with DUS is useful to decrease the use of unnecessary hormones, prevent the loss of sperm doses, and improve the general results of resynchronization programs, shortening the total working period. The simplification of practices, allowing early pregnant ewes to graze longer and preventing unnecessary management – including administration of hormones and insemination – has important welfare implications.

Authors' contributions

IOC collected data, revised and worked on the preparation of the manuscript, and approved the final version. RU proposed the initial hypothesis, organized the study, analyzed the data, and wrote the first draft, revised and worked on the preparation of the manuscript, and approved the final version. MFB discussed the general study design, collected data, revised and worked on the preparation of the manuscript, and approved the final version. EKNA organized the experimental procedures, collected data, revised and worked on the preparation of the manuscript, and approved the final version. JDRS collected data, revised and worked on the preparation of the manuscript, and approved the final version. ABSC collected data, revised and worked on the preparation of the manuscript, and approved the final version. RPC collected data, revised and worked on the preparation of the manuscript, and approved the final version. FZB discussed the general design, collected data, revised and worked on the preparation of the manuscript, and approved the final version.

Conflicts of interest

None of the authors has any conflict of interest to declare.

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