Short Communication

Potential role for GnRH in the synchronization of follicular emergence before the superovulatory Day 0 protocol

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A R T I C L E   I N F O

A B S T R A C T

The ability of gonadotropin-releasing hormone (GnRH) to synchronize ovulation and new follicular wave emergence before a "superovulatory Day 0" protocol was assessed in Santa Inês ewes. For estrus synchronization, a 60-mg medroxyprogesterone acetate sponge was inserted for 6 d. One day before sponge removal, 37.5-µg d-cloprostenol and 300 IU equine chorionic gonadotropin were injected intramuscularly (i.m.). After sponge removal, ewes were assigned to the following 3 groups: (1) G_C—1 mL saline at 12 h (n = 10); (2) G_{24h}—0.025-mg lecirelin (GnRH agonist) i.m. at 24 h (n = 10); or (3) G_{36h}—0.025-mg lecirelin i.m. at 36 h (n = 9). Ovarian ultrasonography was conducted to assess follicular dynamics. Blood was collected to determine plasma concentrations of progesterone and estradiol. Females from G_{36h} and G_C had a greater ($P < 0.05$) estrous response than those from the G_{24h} group (78.0 and 90.0 vs 0.0%, respectively). Ewes from G_{24h} and G_{36h} had earlier ($P < 0.05$) ovulation (48.0 ± 10.2 and 56.7 ± 5.7 h) compared with those from G_c (64.1 ± 9.7 h). The mean number of ovulations per ewe was greater ($P < 0.05$) in G_c (1.9 ± 0.6) and G_{36h} (2.0 ± 1.0) than G_{24h} (1.2 ± 0.4). Plasma concentrations of progesterone and estradiol differed over time. Follicular growth during the postovulatory day was affected ($P < 0.05$) by day of the estrus cycle as well as by the interaction ($P < 0.05$) of treatment and day of the estrus cycle. There was a larger ($P < 0.05$) population of medium follicles during the first 24 h after the ovulation in G_{24h} compared with G_c, and there was an absence of large follicles in G_{36h} between 36 and 72 h after ovulation. In conclusion, the use of GnRH agonist at 36 h more efficiently synchronized ovulation and promoted the absence of dominant follicles during early diestrus and may be used at the start of superovulatory treatment at 80 h in Santa Inês ewes.

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1. Introduction

The use of multiple ovulation and embryo transfer biotechnology in sheep has contributed to rapid genetic growth [1]. However, the response of the donor ewe to gonadotropins during superovulation treatments remains a challenge because of variability in their follicular response [2]. The start of a superovulatory treatment in the absence of follicular dominance is advantageous. The "superovulatory Day 0" protocol was proposed previously as a new method to synchronize ovulation and superovulation onset at Day 0 of the estrus cycle [3]. Its use resulted in better follicular recruitment, greater ovulation numbers, reduced incidence of postovulatory abnormalities [3,4], a greater number of corpora lutea, and improved fertilization rates [5]. In combination, the use of a short-term protocol (5–6 d of progestogen treatment) reduces the time needed between

synchronization and the onset of superovulation, thereby optimizing the multiple ovulation and embryo transfer program [6]. Additionally, studies have demonstrated that the administration of gonadotropin-releasing hormone (GnRH) during mating is an option to improve the endogenous preovulatory luteinizing hormone (LH) surge and ovulatory synchrony to timed artificial insemination protocols [7]. However, the potential for GnRH in synchronizing ovulation and follicular emergence has not been assessed in tropical sheep. Therefore, the aims of this study were to evaluate the ability of a GnRH agonist (lecirelin), administered at 24 or 36 h after a short-term hormonal protocol and synchronize ovulation and follicular emergence in the absence of large follicle(s) before the onset of the superovulatory Day 0 protocol in Santa Inês ewes.

2. Materials and methods

2.1. Location and experimental animals

The study was performed at the Farm School of the Veterinary Faculty at Universidade Federal Fluminense, located in Rio de Janeiro, Brazil (latitude $22°27''$ S). This research was approved by the Animal Care Committee of the university (452/2013) and was conducted under the ethical principles of the Brazilian Society of Laboratory Animal Science. The experiment was conducted during the breeding season (autumn: May, 2013). Multiparous Santa Inês ewes were maintained in a semi-intensive system with access to pasture (*Panicum* sp., and *Brachiaria* sp.) and shelter. Chopped elephant grass (*Pennisetum purpureum*) was offered twice daily, with 300 g/animal of concentrate (17% crude protein) once per day. The animals received water and mineralized salt ad libitum.

2.2. Short-term protocol and GnRH agonist treatment

Intravaginal sponges containing 60 mg of medroxyprogesterone acetate (Progespon; Schering Plough, SP, Brazil) were used for 6 d. One day before sponge removal, 300 IU of equine chorionic gonadotropin (Novormon 5000; MSD Animal Health, SP, Brazil) and 37.5 μg of d-cloprostenol (Prolise; Tecnopec LTDA, SP, Brazil) were administered intramuscularly (i.m.). After sponge removal, the ewes were assigned to the following 3 different groups according to their body weight and body condition score (1–5 scale; [8]): (1) G_C: 1 mL saline i.m. (n = 10) 12 h after sponge removal; (2) G_{24h}: 0.025-mg lecirelin (Gestran Plus; Tecnopec, SP, Brazil) i.m. 24 h after sponge removal (n = 10); or (3) G_{36h}: 0.025-mg lecirelin i.m. 36 h after sponge removal (n = 9). Body weights and body condition score for the 3 groups averaged 50.7 ± 7.3, 48.5 ± 5.1, and 48.5 ± 5.2 kg, and 3.1 ± 0.4, 2.8 ± 0.3, and 3.0 ± 0.3, respectively.

2.3. Ultrasonographic procedures and follicular assessment

Ovarian ultrasonography was conducted every 24 h while the sponges were in place, and every 12 h after removal until ovulation was confirmed. At ovulation (established as Day 0), ultrasonography was performed again every 24 h until Day 5 of the estrus cycle. The examinations were performed using transrectal ultrasonography (SonoScape, Shenzhen, China) equipped with a 7.5-MHz linear transducer with ewes in a standing position. The diameters, positions, and characteristics of the ovarian structures were recorded. The follicles were classified into the following 3 classes according to their size: emerging follicles (≤ 3.0 mm); medium follicles (3–5.0 mm); and dominant follicles (≥ 5.0 mm). The day of ovulation was defined as the day when the largest follicle was no longer detected. The preovulatory follicle diameter was the last measurement obtained.

2.4. Blood collection and hormonal analysis

Blood was collected by jugular venipuncture for 13 d, from sponge insertion to approximately 4 d after ovulation. Blood was collected into tubes with EDTA, and plasma was immediately separated by centrifugation at $1500 \times g$ for 15 min and stored at $-20°C$ until it was analyzed for concentrations of estradiol and progesterone using solid-phase radioimmunoassay kits (Beckman Coulter; Immunotech, Marseille, France). The assay sensitivity and intra-assay coefficients of variation were 2.2 pg/mL and 9% (estradiol), and 0.05 ng/mL and 12% (progesterone). In addition, all data were within the maximum and minimum points of the curve. The cyclicality state of the ewes was determined based on the progesterone concentrations where plasma values ≥ 1.0 ng/mL and <1.0 ng/mL were considered as cyclic and noncyclic ewes, respectively [9].

2.5. Statistical analysis

Descriptive statistics for the reproductive and hormonal data were calculated. In sequence, the Lilliefors test was used to verify the data normality. Parametric data were analyzed using a mixed model procedure for repeated measures, and the Tukey test ($P < 0.05$) was used to compare the means. Nonparametric data were assessed by the chi-square test ($P < 0.05$). The Pearson correlation coefficient was used to compare the hormonal and ultrasound findings. Statistical analyses were performed using Graph-Pad Prism 5.0a software.

3. Results

Data regarding ewe reproductive behaviors are summarized in Table 1. Females from G_{36h} and G_C had a greater ($P < 0.05$) estrous response rate than those in the G_{24h} treatment group. Ewes from the G_{24h} and G_{36h} treatment groups had earlier ovulation ($P < 0.05$) compared with G_C. For the ultrasonography evaluation, values for all variables, with the exception of ovulation rate, differed ($P < 0.05$) among the treatments (Table 1).

Progesterone and estradiol data are shown in Figure 1. Both hormones were affected by day ($P < 0.05$). Plasma progesterone concentrations at sponge insertion revealed that 26 of 29 (89.6%) ewes had a functional corpus luteum (>1 ng/mL). The largest mean progesterone values were detected during the first 3 experimental days (3.03 ± 0.49 ng/mL). The lowest mean progesterone values were

Table 1
Reproductive performance of Santa Inês ewes subjected to short-term synchronization with saline control or GnRH agonist treatment at 24 or 36 h (means ± SEM) to apply in a Day 0 protocol.

Data	Hormonal groups			Mean
	G_c (n = 10)	G_{24h} (n = 10)	G_{36h} (n = 9)	
Reproductive behavior				
Estrus response (%)	90.0 (9/10)[a]	0.0 (0/10)[b]	78.0 (7/9)[a]	53.3 (16/29)
Duration of estrus (h)	37.4 ± 3.9	—	34.3 ± 6.7	36.0 ± 5.3
Interval from sponge removal to onset of estrus (h)	37.3 ± 3.2	—	31.4 ± 3.8	34.8 ± 3.3
Occurrence of ovulation from sponge removal (h)	64.1 ± 3.0[a]	48.0 ± 3.2[b]	56.7 ± 1.9[b]	58.3 ± 3.8
Estrus onset to ovulation (h)	31.0 ± 2.2	—	26.7 ± 3.1	29.1 ± 2.5
Ultrasonography evaluation				
Ovulation rate (%)	100.0[a]	90.0[a]	88.9[a]	96.5 (28/29)
Mean number of ovulations per ewe	1.9 ± 0.2[a]	1.2 ± 0.1[b]	2.0 ± 0.3[a]	1.7 ± 0.3
Largest follicle diameter (mm)	6.5 ± 0.1[a]	5.8 ± 0.2[b]	6.0 ± 0.2[ab]	6.1 ± 0.2
Second largest follicle diameter (mm)	5.5 ± 0.3[a]	4.6 ± 0.2[b]	4.9 ± 0.2[ab]	5.0 ± 0.3

[a, b] Different letters in the same row indicate a significant difference by Tukey test ($P < 0.05$).

observed for the 5-d period after sponge removal (1.11 ± 0.42 ng/mL). In sequence, plasma progesterone increased concomitantly with early formation of the corpus luteum. Plasma concentrations of estradiol were greater ($P < 0.05$) before sponge removal than after sponge removal (Fig. 1).

Follicular growth (small, medium, and large follicles), after the postovulatory day, was affected by the day of the estrus cycle as well as an interaction treatment and day of the estrus cycle (Fig. 2). There was a larger ($P < 0.05$) population of medium follicles during the first 24 h after ovulation in G_{24h} compared with G_c, and there was an absence of large follicles in G_{36h} between 36 and 72 h after ovulation. The concentrations of progesterone were positively correlated (r = −0.23; $P < 0.001$) with the number of dominant follicles. During the first 24 h after sponge removal, there was a positive correlation (r = 0.40; $P < 0.01$) between concentrations of estradiol and the number of emerging follicles. At ovulation, a positive correlation (r = 0.26; $P < 0.05$) was identified between concentrations of estradiol and the number of dominant

follicles. Concurrently, negative correlations were obtained between the number of dominant follicles and the number of emerging follicles (r = −0.28; $P < 0.05$) and median follicles (r = −0.51; $P < 0.01$). During the days after the new follicular wave emergence, a negative correlation was detected between the number of median and dominant follicles (r = 0.40; $P < 0.01$).

4. Discussion

Although a comparison of reproductive variables in the present study to those in previous reports is difficult due to differences in breed, age, nutritional status, and synchronization treatment, our current findings are similar to those reported earlier [10,11]. No ewe from G_{24h} showed estrous behavior, similar to a previous finding in wool sheep treated with GnRH [12]. It is possible that the close proximity of ovulation in this group promoted ovarian and systemic hormonal changes that could not trigger sexual behavior in the central nervous system of the ewes. However, no differences were observed in mean concentrations of plasma
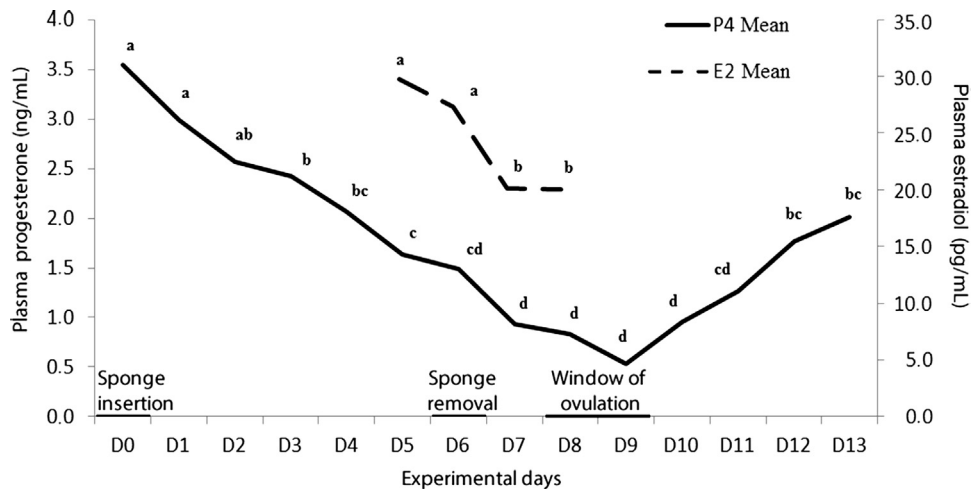

Fig. 1. Mean plasma progesterone and estradiol concentrations in Santa Inês ewes (n = 29) receiving short-term synchronization treatment with saline control or GnRH agonist treatment at 24 or 36 h (means ± SEM) to apply in a Day 0 protocol. [a, b, c, d] Different letters along the line indicate significant difference by Tukey test ($P < 0.05$).

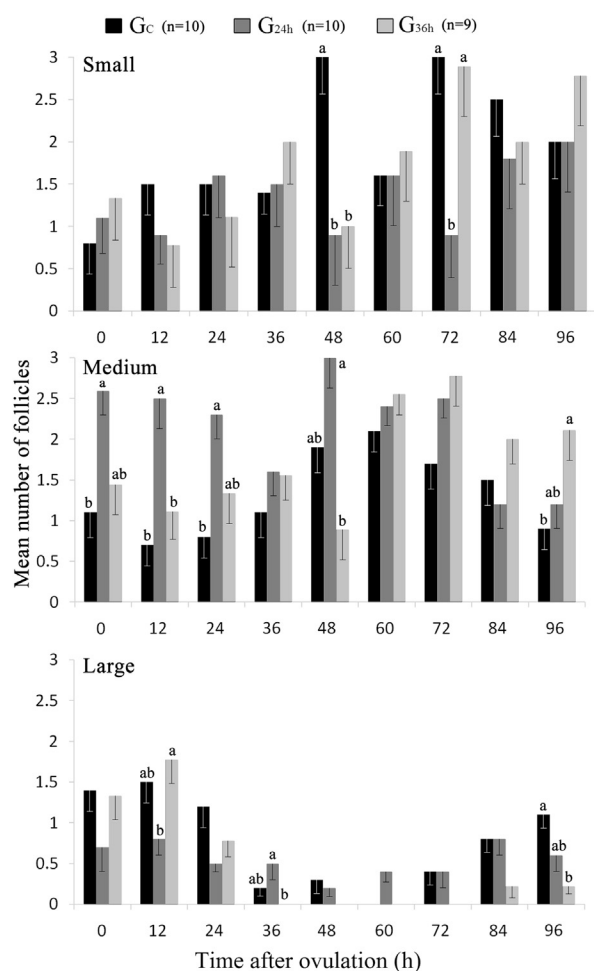**Fig. 2.** Mean number (±SEM) of small (>3 mm), medium (3–5 mm), and large (>5 mm) follicles normalized to the time of ovulation after administration of short-term synchronization with saline control or GnRH agonist treatment at 24 or 36 h (means ± SEM) to apply in a Day 0 protocol in Santa Inês ewes (a vs b: $P < 0.05$).

estradiol for this treatment compared with the other treatments. Both the estradiol increase in the bloodstream and the duration of exposure of estrogen receptors to estradiol in the central nervous system may be associated with estrous expression [13]. Olivera-Muzante et al [14] demonstrated that the administration of GnRH 24 h after sponge removal in synchronization protocols for artificial insemination reduced fertility and fecundity. According to these authors, GnRH administration at this time may promote luteal dysfunction and uterine environments that may not be adequate to sustain embryo development.

GnRH agonist induced ovulation in ewes from both G_{36h} and G_{24h}. However, G_{36h} may have been somewhat more effective in synchronizing ovulation compared with G_{24h} because of the lower standard error of the mean obtained (1.9 vs 3.2). This is a critical time to obtain a population of synchronized follicles in ewe donors before the superovulatory protocol. Although not in estrus, ewes from G_{24h}

had ovulation rates similar to the other ewes but had a lower mean number of ovulations per ewe. In addition, the G_{24h} treatment also showed a reduced number of the largest and second largest follicle diameters compared with G_c. In practice, these findings may be related to lower fertility and prolificacy rates as suggested previously [14].

For the correlation among follicles in different growth phases, Salem et al [15] suggested the possible existence of dominance of dominant follicles over median follicles in sheep. The population of medium follicles was greater in G_{24h} compared with G_c during the first 48 h after ovulation, which may arise from the previous ovulatory follicular wave. However, due to the anticipation of the luteinizing hormone surge, these medium follicles did not reach maturation and probably entered into atresia. After 60 h, the medium follicles from the synchronized follicular wave in G_C and G_{36h} stabilized compared with the follicles from G_{24h}. The largest number of dominant follicles 12 h after ovulation was in G_{36h} compared with G_{24h} and was correlated with the largest number of ovulated follicles in this group. Notably, during the first 96 h of the estrus cycle, G_{36h} presented no dominant follicle between 36 and 72 h after ovulation. Menchaca et al [3] administered GnRH at 36 h after sponge removal in goats and also obtained a small number of dominant follicles at the beginning of the superovulatory treatment. Considering the beneficial effects of G_{36h} in synchronizing ovulation, the optimal time for the start of superovulatory treatment appeared to be at 80 h (56 h for the occurrence of ovulation plus 24 h to be sure about the absence of dominant follicles) for Santa Inês ewes, a tropical hair sheep. Slightly higher values of 84 h have been reported for dairy goats [3] and wool sheep [4].

In conclusion, the administration of a GnRH agonist at 24 h did not have benefits compared with the control group. Conversely, the use of a GnRH agonist at 36 h more efficiently synchronized and promoted the desired environment with the absence of dominant follicles after ovulation. Therefore, the combination of a GnRH agonist at 36 h after sponge removal within the short-term protocol and the start of superovulatory treatment at 80 h for Santa Inês ewes is recommended when using the "Day 0 protocol".

Acknowledgments

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