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Biostimulation with the ram effect increases the follicle recruitment, ovulatory diameter, and embryo viability rate in superovulated ewes

Augusto Ryonosuke Taira [a, *], Felipe Zandonadi Brandão [a], Viviane Lopes Brair [a], Isabel Oliveira Cosentino [a], Felipe Seabra Cardoso Leal [a], Ana Clara Sarzedas Ribeiro [a], Mário Felipe Alvarez Balaro [a], Ribrio Ivan Tavares Pereira Batista [a], Joanna Maria Gonçalves Souza-Fabjan [a], Jeferson Ferreira da Fonseca [b], Rodolfo Ungerfeld [c]

[a] Faculdade de Veterinária, Universidade Federal Fluminense, Av. Vital Brasil Filho, 64, CEP 24230-340, Niterói, RJ, Brazil
[b] Embrapa Caprinos e Ovinos, Núcleo Regional Sudeste, Rodovia MG 133, Km 42, CEP 36155-000, Coronel Pacheco, MG, Brazil
[c] Departamento de Biociencias Veterinarias, Facultad de Veterinaria, Universidad de la República, Lasplaces 1620, Montevideo, 11600, Uruguay

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ABSTRACT

The aim of this study was to compare the preovulatory follicular development and superovulatory outcomes in superovulated ewes which were either stimulated or not stimulated by placement with rams. The treatment regimen to super-stimulate ovarian follicular development was imposed to 28 ewes on "Day 0", from which 14 were stimulated with active rams for 48 h, starting at the time of the fifth FSH dose, with the ram being removed from the pen with the ewes and replaced which other ram every 12 h, using four different rams (group GRE). The other 14 ewes remained isolated from rams throughout the protocol (group GCON). All ewes were administered 133 mg of FSH, into six doses in decreasing quantities, every 12 h. The follicular development and number of ovulations were determined using ultrasonography. Biostimulation resulted in an increased number of large follicles, follicle diameter, and embryo viability rate (viable embryos/recovered structures*100) was greater in ewes of the GRE than GCON group. The number of corpora lutea, follicular cysts, recovered structures, viable embryos, and degenerated and unfertilized structures was similar in ewes of the GRE and GCON group. Structures were recovered from more GRE than GCON ewes. In conclusion, biostimulation with rams during the last phase of the treatment regimen to induce superovulation enhanced the follicular growth and increased the embryo viability rate in ewes.

1. Introduction

An important limitation of superovulatory treatments is that some follicles respond to gonadotrophin stimulation, are recruited, grow, and develop to the size from which there can be an ovulation but ultimately regress without there being an ovulation [1]. This inconsistency between follicle recruitment and ovulation occurrence may be consequence of insufficient pulsatile luteinizing hormone (LH) release from the anterior pituitary. This hormone is a primary factor in modulating the endocrine and morphological changes in the developing follicles and oocytes, including the induction of final maturation, fertilizing capacity, and developmental competence [2]. One strategy to increase the percentage of follicles that grow and from which there is ultimately ovulation and therefore, viable embryo production could be to supplement the LH during this important period of follicular development before there is atresia of these follicles.

No practical treatment regimens have been developed to increase LH exogenous stimulation of follicles by administering LH in ways to mimic the small amplitude LH pulses that induce follicular development and prevent atresia during the proestrus period preceding ovulation. However, there might be natural strategies to increase the secretion of LH. Social stimuli, such as the ram effect, may be an alternative strategy to stimulate LH secretion. The placement of rams with ewes that have not been previously in the presence of rams results in a reduction in the negative feedback

* Corresponding author.
E-mail address: augusto.vete@gmail.com (A.R. Taira).

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effects of estradiol, resulting in an increased frequency of LH pulse release from the anterior pituitary in ewes, does, and cows [3,4]. In anestrous ewes, when there is placement of a ram with anestrous ewes, there is a decrease in estradiol negative feedback inhibition of pulsatile LH release [5]. This management approach has previously been utilized to induce ovulations with their pregnancies resulting in ewes that were previously seasonally [6] or postpartum [7,8] anestrus. Furthermore, the introduction of rams induces an increase of LH pulsatility in cyclic ewes, even during periods when there is ongoing progestogen treatment [9]. This finding is consistent with the observation of an advancement of estrous behavior induced in medroxiprogesterone-pretreated ewes by the introduction of rams, which is probably related to an increase of LH secretion and an increase of the follicular growth rate [10]. Consequently, the placement of rams with ewes results in an increase of LH pulsatile secretion in ovariectomized ewes, whether treated with progestogens or not [11], and in ewes at different stages of the estrous cycle [12]. Therefore, the ram effect has been used to improve the results when there are treatments to synchronize the timing of estrus among ewes in a flock [13,14].

With this in mind, the hypothesis of the present study was that placement of rams with ewes that had not previously been penned with rams would enhance development of follicles and increase the number of ovulations from large ovarian follicles during the period a treatment regimen was being imposed to super-stimulate ovarian follicular development. Therefore, the aim of this study was to compare the follicular development and superovulatory outcomes when there was placement of rams with ewes during the period when a treatment regimen was being imposed to super-stimulate ovarian follicular development.

2. Material and methods

This study was approved by the Ethics Committee for the Use of Animals of the Universidade Federal Fluminense (#95002404/18) and was conducted using procedures consistent with the ethical principles of the Brazilian Society of Science in Laboratory Animals.

2.1. Experimental location, animals, and study design

The research was conducted from November to December (spring), at the Experimental Research Unit in Goats and Sheep (UniPECO) in Cachoeiras de Macacu (22°° 27' S), Rio de Janeiro, Brazil. The study involved 28 multiparous Santa Inês ewes (3.6 ± 1.1 years old; 42.9 ± 4.9 kg; 2.9 ± 0.3 of BCS on a scale of 1–5; mean ± SD). All ewes were subjected to clinical and ultrasonic evaluations and were free from reproductive or clinical disorders. Ewes were managed in an intensive system, fed with chopped Napier grass (*Pennisetum purpureum cv. Cameron*) and 300 g/per animal/daily of concentrate (16% of crude protein), and free access to water and mineral salt (Ovinofós, Tortuga, São Paulo, Brazil).

Experimental ewes were allocated to two groups of 14 ewes each and handled in two repetitions, with seven animals/treatment, separated by one day to ensure that effective and efficient embryo collection processes occurred. The two treatments were 1) ram effect group (group GRE) and 2) control group (group GCON). The stage of the estrous cycles among all the ewes were synchronized using a short-term progestin-based treatment regimen through an intravaginal sponge impregnated with 60 mg of medroxyprogesterone acetate (Progespon; Syntex, Buenos Aires, Argentina) for a duration of six days, plus 0.24 mg of cloprostenol (Estron, Agner Unio, São Paulo, Brazil) i.m., and 300 IU of eCG (Novormon 5000; MSD Animal Health, São Paulo, Brazil) i.m., one day before sponge withdrawal [15]. Thirty-six hours after sponge removal, 0.025 mg of lecirelin i.m. was also administered (Gestran Plus; Tecnopec, São Paulo, Brazil) to synchronize the timing of ovulations among ewes [16].

The treatment regimen to induce superovulations started 80 h after the removal of the sponge (Day 0), with 133 mg of FSH (Folltropin-V, Bioniche Animal Health, Ontario, Canada) i.m. divided into six doses of decreasing quantity as treatments proceeded (33.25/33.25, 19.95/19.95, 13.3/13.3 mg) every 12 h [17]. At the first FSH dose, an intravaginal device containing 0.33 g of progesterone (Eazi-Breed CIDR, Zoetis, São Paulo, Brazil) was inserted into all animals and remained *in situ* until the fifth FSH dose (Day 2). Along with the sixth FSH dose, there was administration of 0.24 mg of cloprostenol i.m. and, 12 h afterwards, 0.025 mg of lecirelin i.m. (Fig. 1).

All ewes were isolated from rams, without visual, olfactory, or auditory stimulus for 60 days before the study began. During all the experimental steps, the ewes assigned to the GCON and GRE group remained in separated barns of the rams (minimum distance = 200 m). While the GCON ewes remained isolated from rams throughout the whole study there was a "teaser" ram placed with the ewes of the GRE group after the progesterone device was removed, at the time of administration of the fifth FSH. For this, four different adult Santa Inês rams (2.9 ± 0.4 years old; 56.8 ± 3.1 kg; 3.0 ± 0.2 of BCS) were used to induce the ram effect. The rams used were reproductively mature, and sexually experienced, as they had been previously utilized for breeding, and subjected to an andrological examination, and were fitted with a protective apron affixed to their abdominal region to prevent copulation. Each ram remained peened with seven ewes, with there being removal and replacement with a novel ram every 12 h thus, there was a total 48 h of biostimulation.

Fresh semen collected from rams with previously proven fertility was deposited into the cervical ostium of all ewes three times. Inseminations were performed 24, 36, and 48 h after the fifth FSH dose, depositing 300 x 10^6 spermatozoa per dose. Cervical insemination was performed with a speculum equipped with a light source and a multidose insemination instrument (Walmur Veterinary Instruments, Montevideo, Uruguay).

2.2. Ultrasonic evaluation

The follicular population was determined using an ultrasonic portable device (Sonoscape S6, Sonoscape, Shenzhen, China) equipped with a 7.5 MHz linear transducer for transrectal use. All scans were performed by the same operator. The ovaries were first evaluated at the beginning of the treatment regimen for inducing superovulation, to assess the ovarian status (Day 0); thereafter, serial assessments were conducted every 12 h from the fifth FSH dose until the last insemination (Day 2 to Day 4). The ovarian ultrasonic evaluations continued every 24 h until the day before embryo collection (Day 5 to Day 9). Follicles were classified based on diameter as small (<3 mm), medium (3–5 mm), or large (>5 mm). The number of follicles in each category was recorded based on the criteria described by Pinto et al. [18]. The Doppler mode was used to assess the corpora lutea, with the following settings: 20% color gain, 10 kHz pulse repetition frequency, 7 cm depth, and 75 kHz wall filter. One day before embryo recovery, the number of corpora lutea and luteal perfusion were determined using Doppler-mode ultrasonography, and only the vascularized corpora lutea were considered functional [19].

2.3. Hormonal treatments for cervical dilation and embryo collection

For embryo collection, all animals were administered the hormonal cervical dilation treatment regimen described by Leite et al.

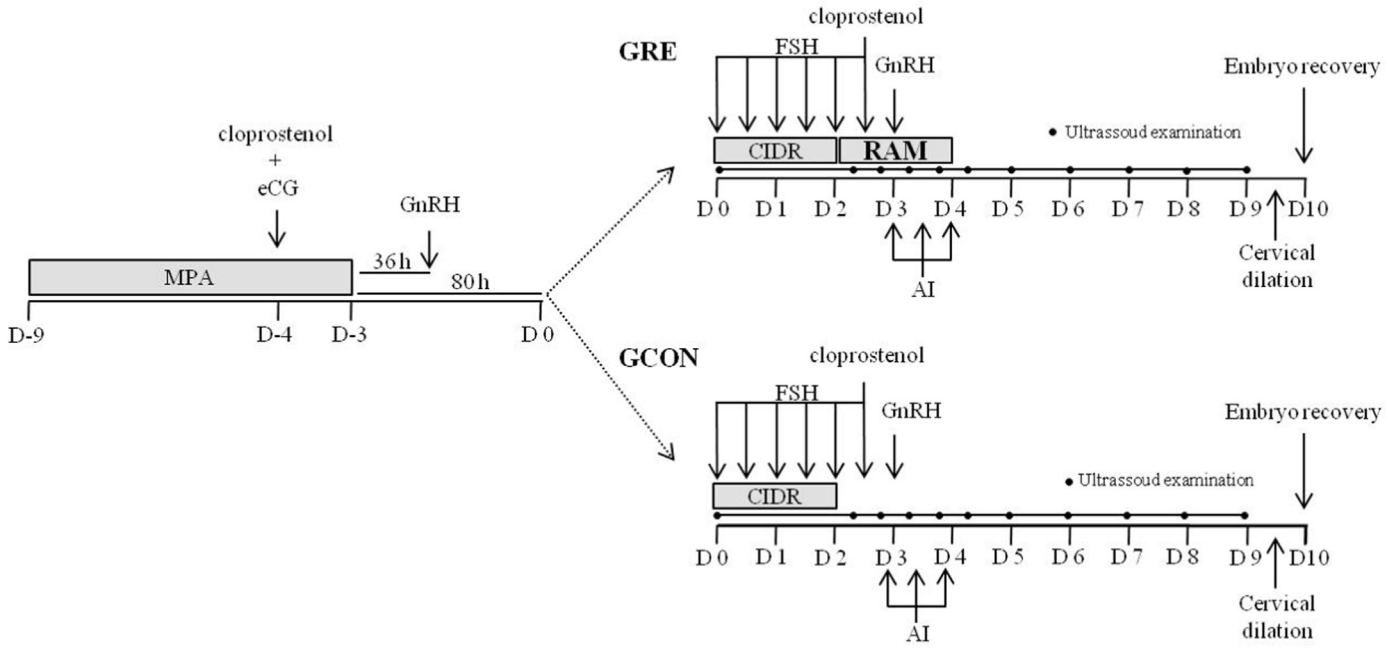

Fig. 1. Experimental procedures and treatments, including hormonal administration, period of ram stimulation, ultrasonic evaluation, and embryo recovery; MPA: medroxyprogesterone acetate; CIDR: progesterone device; eCG: equine Chorionic Gonadotropin; FSH: follicle stimulating hormone; AI: artificial insemination; Experimental groups: GCON – superovulated ewes which remained isolated from rams; GRE – superovulated ewes where there was placement of rams with ewes during the latter part of the period when the hormonal treatment regimen was imposed.

[20]: 100 μg of estradiol benzoate i.v. (RIC-BE, Agener Union, São Paulo, Brazil) diluted in 2.5 mL of absolute ethyl alcohol and 2.5 mL of saline, and 0.12 mg of cloprostenol i.m., 12 h before embryo collection. In addition, 100 IU of oxytocin i.v. (Oxytocin Forte UCB; Centrovet, Goiânia, Brazil) was administered 15 min before the embryo collection procedure was initiated.

Ewes were sedated with 0.1 mg/kg of acepromazine maleate i.v. (Acepran; Vetnil, São Paulo, Brazil) and 0.3 mg/kg of diazepam i.v. (Diazepam; Santisa, São Paulo, Brazil), in addition to epidural anesthesia with 2.0 mg/kg of ketamine hydrochloride (Cetamin; Syntec, São Paulo, Brazil) [20]. Embryo collection was performed immediately, after cervical traction and fixation. The structures were recovered using a closed-circuit system (Embrapa Circuit for the recovery of goat/sheep embryos; Embrapa, Brazilia, Brazil), using the procedure described by Fonseca et al. [21].

After this procedure, the total structures (embryos, unfertilized oocytes, empty pellucid zones, and degenerated structures) were screened and counted in a stereomicroscope. The embryos were classified according to stage of development and morphological characteristics, as described by IETS [22]. Only class 1 embryos (grade I, II, and II) were considered viable embryos.

2.4. Blood collection and progesterone quantification

Six blood samples were collected every 24 h from Day 4 to Day 9 (Fig. 1) using jugular venipuncture procedures. Blood samples were centrifuged at 1500 *g* for 15 min, and the serum was separated and immediately stored at −20 °C until the progesterone assay was performed. Progesterone values were determined using a solid-phase radioimmunoassay utilizing a commercial kit (MP Diagnostics Division, Orangeburg, New York, USA) [23]. Samples were analyzed in a single assay, with a sensitivity of 0.05 ng/mL and an intra-assay coefficient of variation of 8.9%, with all values within the curve.

2.5. Statistical analysis

The numbers of small, medium, and large follicles were compared with a mixed model, including treatment, time, and their interaction as the main effects, considering time as a repeated measure. The embryo recovery rate was calculated as recovered structures*100/(number of corpora lutea); the viability rate as viable embryos*100/(recovered structures); and the unfertilized rate (and rate of degenerates) as unfertilized structures*100/(recovered structures). The percentage of large follicles from which there were ovulations was calculated as the number of corpora lutea*100/(maximum number of large follicles observed using ultrasonic procedures). The luteal functionality was evaluated and compared with results when using B-mode and Doppler-mode ultrasonic procedures. The data were compared with a mixed model (SAS University Edition). The model included the treatments (GCON and GRE) as main factors and the repetition as a random factor. These results are expressed as LSmeans ± SEM. The proportion of ewes from which at least one structure was recovered was compared with the Fisher exact probability test. The dispersion of the proportion of large follicles from which there were ovulations was compared using the Bartlett test. There were considered to be mean differences when there was a $P \leq 0.05$, and there were considered to be trends for mean differences when there was $0.05 < P < 0.1$.

3. Results

3.1. Follicular population

The number of follicles from all categories of follicles (small, medium, and large) varied according to the treatment, time, and an interaction between treatment and time ($P < 0.0001$ for the three factors in all the follicular categories). At the beginning of the treatment regimen to induce superovulation (Day 0), the

population of small, medium, and large follicles was similar between groups; however, after the placement of the rams with the ewes (fifth FSH dose; Day 2) until Day 3.5, ewes of the GRE group had more large follicles than GCON ewes (Fig. 2). The number of small and medium follicles was greater in ewes of the GCON than GRE group until Day 4 and Day 5, respectively.

The diameter of the largest and second-largest follicles before ovulation was greater in ewes of the GRE than GCON group ($P < 0.0001$ and $P = 0.002$, respectively). There were the largest number of large follicles at the same time points in ewes of both

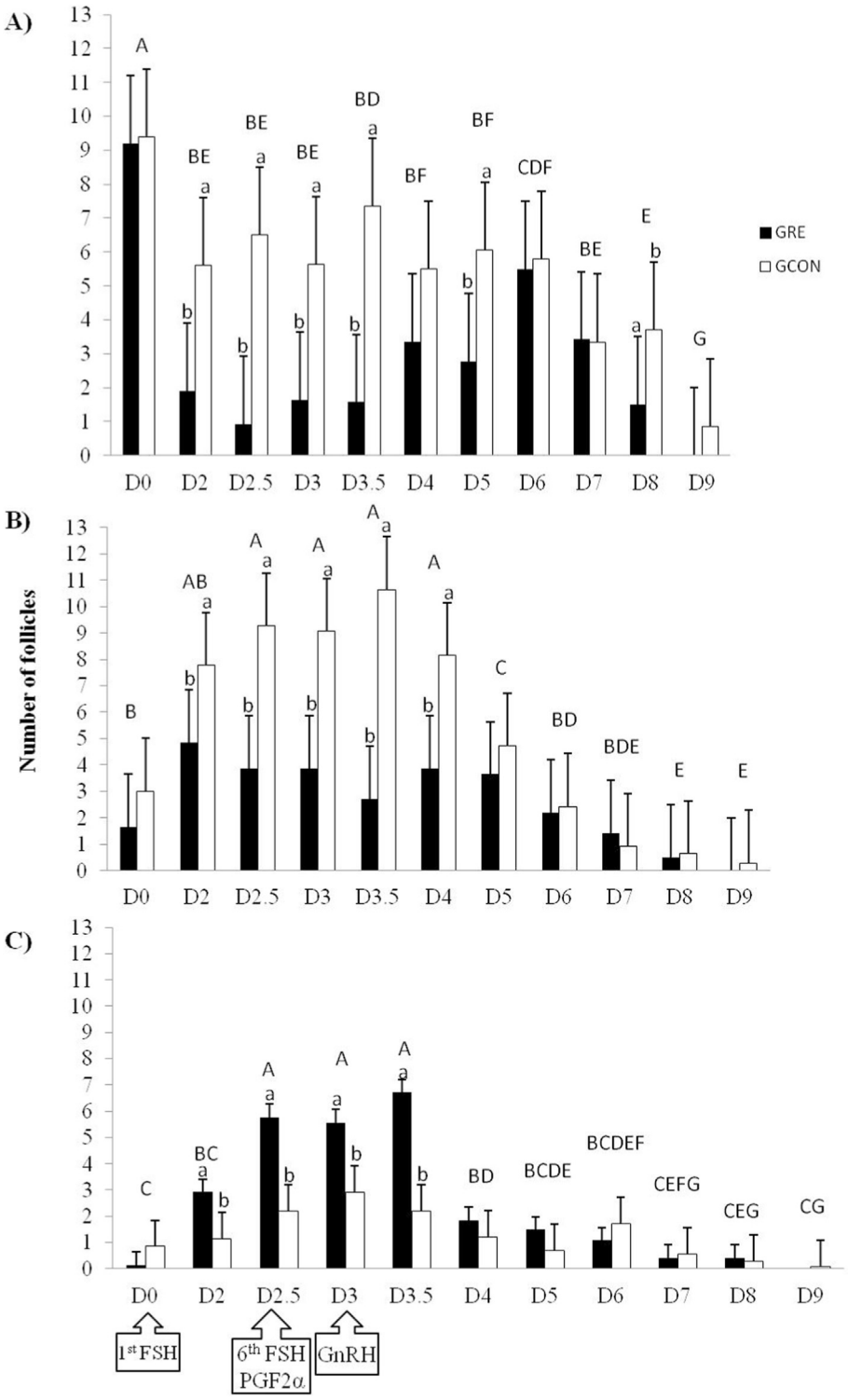

Fig. 2. Number of A) small (<3 mm), B) medium (3–5 mm), and C) large (>5 mm) follicles throughout the period when the hormonal treatment regimen was imposed, based on the "Day 0" protocol in superovulated ewes where there was placement of rams with ewes (GRE; black bars) and superovulated ewes which remained isolated from rams (GCON; white bars). The timing of FSH, GnRH, and cloprostenol administration is indicated with arrows. Different capital letters indicate differences over time ($P < 0.05$). Different lower-case letters indicate differences between groups at the same time point ($P < 0.05$).

groups. Although the number of large follicles was greater in the ewes of the GRE than GCON group (P = 0.001; Table 1), the proportion of large follicles from which there were ovulations was greater in ewes of the GCON group (P = 0.02; Table 1). However, the proportion of large follicles from which there were ovulations was more homogeneous in the ewes of the GRE than GCON group (P = 0.009).

3.2. Ovarian response and embryo production

The luteal perfusion assessed using Doppler procedures, as well as the number of corpora lutea, viable embryos/number of corpora lutea, and anovulatory follicles did not differ between groups. Similarly, the percentage embryo recovery, number of viable embryos, and total number of recovered, degenerated, and unfertilized structures per ewe did not differ between groups. Nonetheless, more ewes of the GRE group had at least one structure recovered than ewes of the GCON group, furthermore, ewes of the GRE group had a higher viability rate (viable embryos/recovered structures) (P = 0.02 and P = 0.05 respectively; Table 2).

3.3. Serum progesterone concentrations

There were no differences in P4 concentrations as a result of treatment with concentrations only varying with time (P < 0.0001). The values increased from Day 4–7 (0.02 ± 0.45 compared with 2.52 ± 0.45 ng/mL, respectively; P < 0.0001), on Day 8 (4.24 ± 0.45; P = 0.0009), and from Day 8–9 (6.33 ± 0.45; P < 0.0001).

4. Discussion

The main outcomes in the present study confirm the hypothesis, because the placement of reproductively mature rams during the last phase of the superovulatory protocol resulted in a greater the number of large follicles, larger diameter at the time of ovulation, and increased embryonic viability rate. Although the number of corpora lutea and concentrations of P4 were similar between ewes of the two groups, the results indicate that biostimulation could be an effective procedure to stimulate the development of a larger number of follicles as a result of treatments to stimulate follicular development than occurred in the ewes where there was no biostimulation. Furthermore, the number of ewes from which oocytes/embryonic structures were recovered was larger than in the ewes for which there was no biostimulation imposed, and some outcomes were more homogeneous among ewes of the group in which there was biostimulation imposed. This is a positive outcome, considering the large amount of variability that often occurs in the response to hormonal treatment regimens for inducing superovulation responses [24]. It remains to be determined how this stimulation could also cause an increase in the number of follicles from which there is ovulation; however, increasing the follicular recruitment and embryo quality are key results in any strategy to improve the results of treatments to super-stimulate follicular development.

The placement of rams with ewes induced an increase in the number of large follicles. This was probably a consequence of the stimulation of the growth of small and medium follicles because these results occurred simultaneously. The results from the present study indicate the decrease of small and medium follicles was due to the greater growth rate in ewes where there was biostimulation with rams of ovarian follicles [25,26], which probably is the reason for the greater follicle growth, as previously reported in the same breed [27]. The greater follicular growth rate in ewes in which there was biostimulation is probably a consequence of the rapid response to gonadotropic stimulation. The strategy of exchanging the "teaser" ram every 12 h was probably critical as it repeatedly renewed the stimulus during the 48-h period when rams were with the ewes because ram sexual behavior is related extent of biostimulation that results when rams are placed with ewes, including the luteal function [28]. It is important to ensure that the biostimulation effects are sufficient for inducing the endocrine/physiological responses, which can be enhanced after a period when there is no ram penned with the ewes. Similarly, it is interesting to hypothesize the possible effects of changing the length of the stimulation period, because prolonging the period rams are penned with ewes might be a strategy to increase the proportion of large follicles from which there are ovulations.

In the present study, biostimulation also had positive effects on embryo quality, a result of the greater percentage of viable embryos. There may be several explanations for this finding, which are not mutually contradictory. First, the proportion of viable oocytes with fertilization capacity is related to the size of the follicles from which there is ovulation [29]. In this sense, the estradiol:progesterone ratio of the largest follicles results in the oocytes contained in these follicles having greater developmental competence than those from smaller follicles, resulting in a greater percentage blastocyst rate after *in vitro* fertilization [30]. Developmental competence is also related to the capacity for nuclear and cytoplasmic maturation, because the pre-ovulatory surge release of LH induces the resumption of meiosis by modulating oocyte development so that there is competence for fertilization [2,31]. Crozet et al. [32] reported similar results in goats, where the percentage of morula or blastocyst produced *in vitro* was related to the follicular size at the time of ovulation. However, it is possible that the difference in the preovulatory dynamics led to modification of the oviductal–uterine milieu; interestingly, however, this was unrelated to the corpora lutea function and/or progesterone concentrations, ensuring there was an adequate uterine milieu present for embryo development. In conclusion, the biostimulation by placing rams with ewes during the latter portion of the treatment regimen

Table 1
Ultrasonographic outcomes from Santa Inês ewes in which there was superovulation with FSH (133 mg in six decreasing doses) after "Day 0" protocol, when there was or was not placement of rams with ewes during the superovulatory treatment regimen. The data are expressed as LSmeans ± SEM.

	GCON	GRE	P
Maximum number of large follicles	4.1 ± 0.7	7.6 ± 0.7	0.001
Moment at which the maximum number of large follicles was observed (day of treatment)	4.4 ± 0.9	4.5 ± 0.9	ns
Largest follicle (mm)	5.9 ± 1.3	7.6 ± 1.5	<0.0001
Second largest follicle (mm)	5.2 ± 1.6	6.4 ± 0.8	0.002
Large follicles that ovulated (%)[a]	208.8 ± 133.3	118.2 ± 27.6	0.02

GCON: superovulated ewes which remained isolated from rams the period the treatment regimen was imposed.
GRE: superovulated ewes where there was placement of rams with ewes during the treatment the period the treatment regimen was imposed.
Large follicles that ovulated: number of corpora lutea*100/maximum number of large follicles.
ns: not significant.
[a] The dispersion of the data differed (P = 0.009).

Table 2
Ovarian response and embryo production in Santa Inês ewes treated to super-stimulate ovarian follicular development with FSH (133 mg in six decreasing doses), where there was or was not placement of rams with ewes (GRE and GCON, respectively) during the period when the treatment regimen was imposed to super-stimulate follicular development in ewes that were subjected to non-surgical embryo recovery.

	GCON	GRE	P
Ewes washed with at last one structure recovered (%)	6/14 (42.9)	12/14 (85.7)	0.02
Number of corpora lutea	8.0 ± 1.0	9.1 ± 1.0	ns
Number of anovulatory follicles	1.2 ± 0.4	0.6 ± 0.4	ns
Recovered structures	3.0 ± 0.9	3.9 ± 0.9	ns
Number of viable embryos	2.2 ± 0.9	3.0 ± 0.8	ns
Number of unfertilized structures	0.8 ± 0.5	0.8 ± 0.5	ns
Number of degenerate structures	0.01 ± 0.1	0.08 ± 0.1	ns
Recovery rate (%)	31.3 ± 13.6	49.3 ± 13.3	ns
Viability rate (%)	36.7 ± 13.0	73.8 ± 12.4	0.05
Unfertilized rate (%)	13.3 ± 9.9	16.9 ± 9.5	ns
Degenerate rate (%)	0.01 ± 1.2	1.5 ± 1.2	ns

The data are expressed as LSmean ± SEM.
GCON: superovulated ewes which remained isolated from rams the period the treatment regimen was imposed.
GRE: superovulated ewes where there was placement of rams with ewes during the treatment the period the treatment regimen was imposed.
Recovery rate: recovered structures*100/number of corpora lutea.
Viability rate: viable embryos*100/recovered structures (oocytes or embryos).
Unfertilized rate: unfertilized structures*100/recovered structures (oocytes or embryos).
Degenerate rate: degenerated structures*100/recovered structures (oocytes or embryos).
ns: not significant.

for super-stimulation of ovarian follicular development resulted in the stimulation of follicular growth and increased the percentage embryonic viability in ewes.

CRediT authorship contribution statement

Augusto Ryonosuke Taira: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Felipe Zandonadi Brandão:** Project administration, Supervision, Resources, Visualization, Conceptualization, Methodology. **Viviane Lopes Brair:** Investigation, Data curation, Writing – review & editing. **Isabel Oliveira Cosentino:** Investigation, Data curation. **Felipe Seabra Cardoso Leal:** Investigation, Data curation. **Ana Clara Sarzedas Ribeiro:** Investigation, Data curation. **Mário Felipe Alvarez Balaro:** Investigation, Data curation, Writing – review & editing. **Ribrio Ivan Tavares Pereira Batista:** Investigation, Data curation. **Joanna Maria Gonçalves Souza-Fabjan:** Investigation, Data curation, Writing – review & editing. **Jeferson Ferreira da Fonseca:** Writing – review & editing. **Rodolfo Ungerfeld:** Project administration, Supervision, Resources, Visualization, Conceptualization, Methodology, Writing – review & editing.

Declaration of competing interest

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

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