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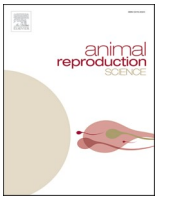

The pFSH dose affects the efficiency of *in vivo* embryo production in Santa Inês ewes

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ABSTRACT

This study compared the follicular growth, superovulatory response, and *in vivo* embryo production after administering two doses of porcine follicle-stimulating hormone (pFSH) in Santa Inês ewes. The estrous cycle of 36 multiparous ewes was synchronized with the Day 0 protocol and superovulated with 133 mg (G133, n=18) or 200 mg (G200, n=18) of pFSH. Ultrasonographic evaluations of the ovaries were performed, ewes were mated and submitted to non-surgical embryo recovery. Viable blastocysts were stained with Nile Red and Hoechst. The G200 had a greater number of medium and large follicles, as well as a larger size of the third largest follicle. A total of 97.2% (35/36) of the ewes came into estrus and it was possible to transpose cervix in 80.6% (29/36). There were no effects of treatments in the response to superovulation, the proportion of ewes in which was possible to transpose the cervix, the number of corpora lutea, the number of anovulatory follicles, the proportion of ewes flushed with at least one recovered structure, number of recovered structures, number of viable embryos, viability rate, and recovery rate. The G200 ewes were in estrus for a longer period of time than the G133 ewes (54.0 ± 4.5 h *vs.* 40.3 ± 3.6 h) and produced more freezable embryos (6.5 ± 1.6 *vs.* 2.3 ± 0.7) than G133. Both doses promoted an efficient superovulatory response and did not affect embryonic lipid accumulation. The dose of 200 mg of pFSH showed greater potential to increase the superovulatory response, as it increased follicular recruitment and the recovery of freezable embryos.

1. Introduction

Multiple ovulation and embryo transfer (MOET) has contributed to genetic, zootechnical and sanitary improvement programs for small ruminants around the world, as well as to the rescue and conservation of endangered breeds, the import and export of germplasm

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and in supporting other related reproductive biotechniques (Cognié et al., 2003; Menchaca et al., 2010). In recent years, MOET in sheep had a relevant increase in the number of embryos produced and transferred, performed mostly with fresh embryos (90.0%) (Viana, 2022). Despite the advances, this biotechnology still has some obstacles that limit its massive application in sheep, especially in commercial conditions.

Superovulation (SOV) is a major challenge within MOET programs and has been the focus of studies due to the high variability of results and cost of the protocols (Bartlewski et al., 2016; Pinto et al., 2017). Superovulatory treatments are based on the use of high doses of gonadotropins, which stimulate the synchronous growth of several antral follicles, resulting in multiple ovulations. Follicle-stimulating hormone (FSH) is the gonadotropin of first choice in sheep (Khan et al., 2022), with great variation between protocols in terms of origin and dosage, with inconstant responses in different breeds (Wu et al., 2011; Rodriguez et al., 2019; Dias et al., 2023). For example, Gibbons et al. (2010) and Loiola Filho et al. (2015) obtained similar superovulatory responses using 200 mg of porcine FSH (pFSH) or 80 mg in Merino ewes and 128 mg in Dorper ewes, respectively. In Lacaune ewes, treatment with 200 mg pFSH induced a greater superovulatory response than a reduced dose of 100 mg (Figueira et al., 2020). Moreover, in Santa Inês, Maciel et al. (2019) reported a worse superovulatory response using 133 mg of pFSH than 100 mg or 200 mg doses, whose answers were similar.

In other species, the FSH dose can have controversial effects on embryo production. For example, in humans, FSH dose is negatively related to the number of oocytes (Clark et al., 2021) and embryos (Luo et al., 2022) recovered, and to the live birth rate after embryo transfer (Baker et al., 2015; Munch et al., 2017; Shaia et al., 2020). In mice, high doses of FSH *in vivo* or *in vitro* can interfere with the development of oocytes and the resulting embryos, despite not influencing the physiology and behavior after the birth of the offspring (Li et al., 2013).

Ovarian follicular status at the start of superovulatory treatment also affects embryo production, being positively influenced by a higher antral follicle count and absence of large follicles (Gonzalez-Bulnes et al., 2002; Veiga-Lopez et al., 2005). Thus, Menchaca et al. (2007), (2009) proposed to perform a short protocol for estrus synchronization to promote synchronous ovulation before beginning the SOV treatment. As the first wave of follicular growth begins shortly after ovulation, this is the optimum time to begin the SOV (day 0 of the estrous cycle). Balaro et al. (2016) confirmed the applicability of the "Day 0 protocol" in Santa Inês ewes, and its use has been shown to be efficient in the *in vivo* production of embryos in these donors (Pinto et al., 2020, 2018; Santos et al., 2020; Taira et al., 2022).

Considering the individual response of the breeds and the great variation in the protocols used in the MOET, the present study aimed to compare the follicular development, the *in vivo* embryo production, and the embryo quality as well as their lipid content in Santa Inês ewes treated with 133 mg or 200 mg of pFSH subjected to the Day 0 superovulatory protocol.

2. Material and methods

2.1. Experimental location, animals, and experimental design

The project was approved by the Ethics in Animal Use Committee of Universidade Federal Fluminense (7878010521–ID001136) and was conducted following the ARRIVE guidelines (Animal Research: Reporting of *In Vivo* Experiments). The study was carried out in the spring, at the 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. A total of 36 multiparous Santa Inês ewes (3.9 ± 0.9 years; 52.8 ± 10.5 kg; 3.0 ± 0.3 body condition score on a scale of 1–5; mean ± SD) were used. This breed has only slight seasonal changes at this location (Balaro et al., 2014), so all ewes were cycling, as evidenced by the presence of at least one corpus luteum (CL) at the beginning of the hormonal protocol. All animals were at least eight months postpartum, and were previously submitted to clinical and ultrasonographic evaluation, and only those healthy and free of reproductive disorders were used. Ewes were handled in an intensive system, fed with elephant grass (*Pennisetum purpureum* Schum.), 300 g/animal/day of concentrate (16% crude protein), and free access to water and mineral salt (Salminas, Nutriplan, Juiz de Fora, Brazil). The animals were allocated to two experimental groups and administered a

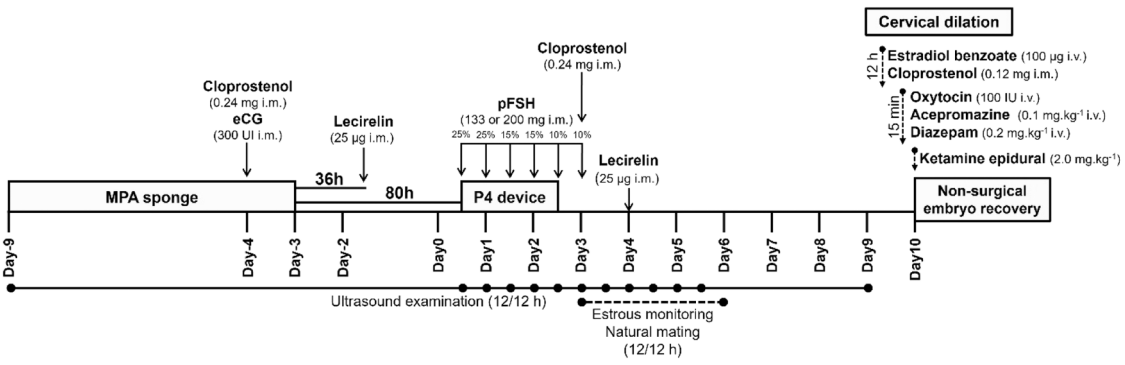

Fig. 1. Experimental design for *in vivo* embryo production in Santa Inês ewes. MPA: medroxyprogesterone acetate; P4: progesterone; pFSH: porcine follicle-stimulating hormone; eCG: equine chorionic gonadotropin.

total dose of either 133 mg/ewe (G133; n=18) or 200 mg/ewe (G200; n=18). The groups were homogeneous in body weight and body condition score. As it was not possible to record all the data in all ewes simultaneously, treatments were applied in five replicates of 7–8 ewes/each, separating one day for each replica. Three or four ewes from each group were included in each replica. The experimental activities are described in detail in Fig. 1.

2.2. Estrous synchronization treatment, superovulation, and mating

The ewes were submitted to a short estrous synchronization protocol proposed by Balaro et al. (2016), following the Day 0 protocol (Menchaca et al., 2009). Briefly, an intravaginal sponge impregnated with medroxyprogesterone acetate (60 mg; Progespon; Zoetis, Campinas, Brazil) was inserted, remaining *in situ* for six days. Twenty-four hours before sponge removal, eCG (300 IU i.m.; Novormon; Zoetis, Campinas, Brazil) and sodium cloprostenol (0.24 mg i.m.; Estron, Agner União Saúde Animal, São Paulo, Brazil) were administered. Females received lecirelin 36 h after sponge removal (25 μg i.m.; TEC-Relin, Agener União Saúde Animal, São Paulo, Brazil). Ewes were superovulated with 133 mg or 200 mg of pFSH i.m. (Folltropin-V, Vetoquinol, Lavaltrie, Canada) in six decreasing doses (25%, 25%, 15%, 15%, 10%, and 10% of the total dose), every 12 h, with the first dose given 80 h after removal of the intravaginal sponge. At this time, a new intravaginal progesterone implant (P4; 0.36 g; Primer PR, Agener União Saúde Animal, São Paulo, Brazil) was inserted, remaining *in situ* until the fifth dose of pFSH. Simultaneously with the last dose of pFSH, sodium cloprostenol (0.24 mg i.m.) was administered, and after 24 h the sheep received lecirelin (25 μg i.m.). All ewes were checked for estrous behavior and mated naturally every 12 h, from the sixth dose of pFSH until the end of estrus. For that, rams with proven fertility by andrological examination were used.

2.3. Ultrasound evaluations

Follicular populations were determined using a portable ultrasound device (Sonoscape S6, Sonoscape, Shenzhen, China) with a 7.5 MHz transrectal linear transducer. All assessments were performed by the same operator. The follicular population was evaluated every 12 h, from the first dose of pFSH until 60 h after the last dose, totaling 11 moments of recordings (Day 0.5 to Day 5.5). 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. (2020). One day before embryo collection, ovarian status and the number of vascularized CLs were determined, using B-mode and color Doppler ultrasonography according to Pinto et al. (2018). Luteal vascularization was subjectively assessed using an increasing score scale of 1–4, based on the luteal area percentage (0%–100%) with colored pixels, and only functional CL (score $\geq$ 2) in each ovary were counted. Animals with $\geq$3 CLs were considered responsive to SOV.

2.4. Cervical dilation and non-surgical embryo recovery

Non-surgical embryo recovery (NSER) was performed on Day 10. The animals were previously submitted to a hormonal protocol for cervical dilation (Leite et al., 2018), administering of estradiol benzoate (100 μg i.v.; RIC-BE, Agener União Saúde Animal, São Paulo, Brazil), diluted with 2.5 mL of absolute ethyl alcohol and 2.5 mL of saline solution, 12 h before embryo collection, simultaneously to the administration of cloprostenol sodium (0.12 mg i.m.). In addition, oxytocin (100 IU i.v.; Ocitocina Forte UCB, Centrovet, Goiânia, Brazil) was administered 15 min before embryo collection. The animals were sedated with acepromazine maleate (0.1 mg.kg-1 i.v.; Acepran, Vetnil, Louveira, Brazil) and diazepam (0.2 mg.kg-1 i.v.; Diazepam, Teuto, Anápolis, Brazil), and submitted to epidural anesthesia with ketamine hydrochloride (2.0 mg.kg-1; Cetamin, Syntec, Barueri, Brazil). For NSER, fixation and traction of the cervix were performed, followed by washing of the uterine horns and recovery of the structures by a closed circuit (Embrapa Circuit for recovery of goat/sheep embryos, Embrapa, Brasília, Brazil), according to Fonseca et al. (2013). The recovered structures were sorted and counted under a stereomicroscope. The collected embryos were classified according to developmental stage and morphological characteristics, as described by the International Embryo Technology Society (IETS) (Mapletoft et al., 2020). Grade 1, 2, and 3 embryos were considered viable, and grade 1 and 2 embryos were freezable. A subsample of viable blastocysts, each recovered from different ewes in each group (G133: n=7; G200: n=10) was randomly selected during the experiment for lipid quantification (see Section 2.5).

2.5. Lipid quantification

Embryos were fixed in a 500 μL solution of 4% paraformaldehyde for at least 24 h. The quantification of lipids was performed according to the methodology described by Batista et al. (2014). The fixed embryos from each group were transferred to a four-well microplate containing 500 μL of 10 μg/mL Nile Red (Sigma-Aldrich Corporation, Saint Louis, USA). Samples were stained in the dark at room temperature for 24 h. Nile Red stock solution (1 mg.mL-1) was prepared by dilution in dimethylsulfoxide (DMSO) and stored at room temperature in the dark, and the final concentration was obtained by diluting the stock with phosphate-buffered saline (PBS) with 0.4% bovine serum albumin (BSA). To determine the number of blastomeres per embryo, the embryos were incubated in Hoechst 33342 (1 mg.mL-1) at room temperature for 5 min. The slides were prepared, and each sample (embryo) was evaluated, for Nile Red (excitation: 530 nm; emission: 635 nm) and Hoechst (excitation: 350/461; emission: 461 nm), in an epifluorescence microscope (Nikon Eclipse Ci, Nikon Corporation, Tokyo, JPN) with 10x objective. A UV light filter was used to prevent bleaching. In the images obtained from each embryo, fluorescence was quantified using the ZEN 3.5 Software, and the results were expressed in arbitrary

fluorescence units; and the total number of blastomeres, using the QuPath 0.3.2 Software.

2.6. Statistical analysis

Data analysis was performed using the SAS statistical software (SAS on Demand for Academics). The numbers of small, medium, and large follicles; and the size of the largest follicles were compared by a mixed model, including the treatment, time, and their interaction as main effects, considering time as a repeated measure. The replicate was included as a random factor. The proportions of ewes that came into estrus, ewes in which was possible to transpose the cervix, ewes responsive to the treatment ($\geq$ 3 CLs), and ewes from which at least one structure was recovered, were compared using the Fisher's Exact Test. The other variables were tested for normality using the Shapiro-Wilk test. The Mann-Whitney test was used to compare the onset and duration of estrus, the number of CLs, the number of anovulatory follicles, the number of recovered structures per ewe, the viable embryos, the recovery and viability rates, the number of blastomeres per embryo, and the proportions of blastocysts/embryos and morulaes/embryos. The number of freezable embryos and the quantification of lipids per embryo were compared using a mixed model, including the treatments as the main effect and the replication as a random factor. The embryo recovery rate was calculated as recovered structures*100/number of CLs, and the viability rate as viable embryos*100/recovered structures. Results are presented as LS mean $\pm$ SEM or mean $\pm$ SEM according to the test used. A difference between means was considered significant when $P \leq 0.05$, and a tendency when $0.05 < P \leq 0.1$.

3. Results

3.1. Follicular population

The number of small (G133: 2.4 $\pm$ 0.2 *vs.* G200: 2.3 $\pm$0.2), medium (G133: 5.4 $\pm$ 0.2 *vs.* G200: 7.2 $\pm$ 0.3) and large follicles (G133:

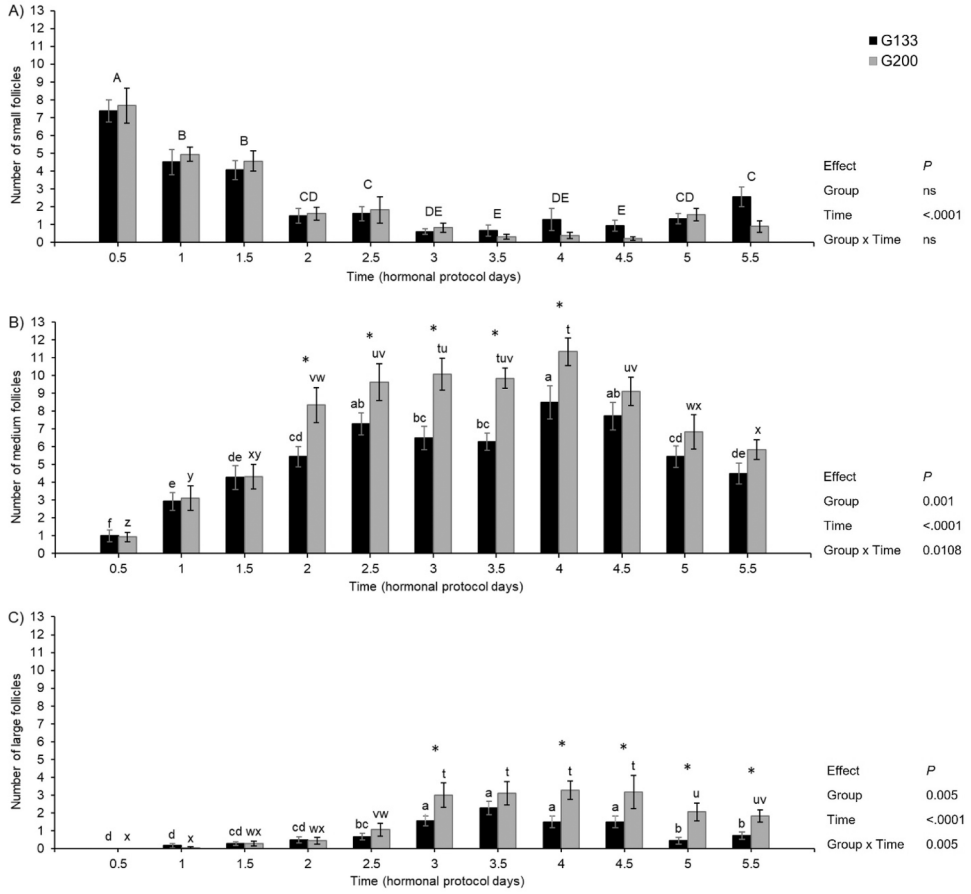

Fig. 2. Number of A) small (<3 mm), B) medium (3–5 mm), and C) large (>5 mm) follicles during superovulatory treatment with a total dose of 133 mg (G133, black) or 200 mg (G200, gray) of pFSH, based on Day 0 protocol in Santa Inês ewes. Follicular status was assessed by B-mode transrectal ultrasound every 12 h from the first dose of pFSH until 36 h after lecirelin administration (Day 0.5 to Day 5.5). Different capital letters indicate differences over time ($P < 0.05$). Different lowercase letters indicate differences over time for each treatment (G133: a, b, c, d, e, f; G200: t, u, v, w, x, y, z; $P < 0.05$). *Indicates differences between groups at the same assessment time ($P < 0.05$). ns: not significant.

0.9 ± 0.1 *vs.* G200: 1.7 ± 0.2) observed in both groups are presented in Fig. 2. The number of medium and large follicles was greater in G200 than G133 (P=0.001 and P=0.005, respectively), with no effect on the number of small follicles. The number of follicles from the three categories varied with time (P<0.0001 for the three comparisons): regardless of the experimental group, there was a progressive increase in the population of medium and large follicles, accompanied by a decrease in the number of small follicles during the study period. There was a significant interaction between treatment and time in the number of medium and large follicles (P=0.01 and P=0.005, respectively). The number of medium follicles was greater in G200 than G133 from time Day 2 to Day 4 (P<0.05). At the time of the first pFSH dose, there were no large follicles in any of the groups, but the number of these follicles was greater in G200 than in G133 on Day 3 and Day 4–Day 5.5 (P<0.05).

The size of the first (G133: 4.8 ± 0.1 *vs.* G200: 5.2 ± 0.1 mm), second (G133: 4.0 ± 0.1 *vs.* G200: 4.2 ± 0.1 mm) and third (G133:3.4 ± 0.1 *vs.* G200: 3.7 ± 0.1 mm) largest follicles of the experimental groups is presented in Fig. 3. The treatments did not modify the diameter of the first and second largest follicles, but the diameter of the third largest follicle tended to be greater in G200 than G133 (P=0.1). The diameter of the greater three follicles varied with time (P<0.0001 for the three), and there was an interaction between treatment and time (P=0.004, P=0.03, and P=0.03, respectively). The diameter of the largest follicle was greater in G200 than G133 at Days 4, 5, and 5.5; that of the second largest follicle at Days 1, 5, and 5.5; and the diameter of the third largest follicle at Days 3, 5 and 5.5 (P<0.05 in all the comparisons).

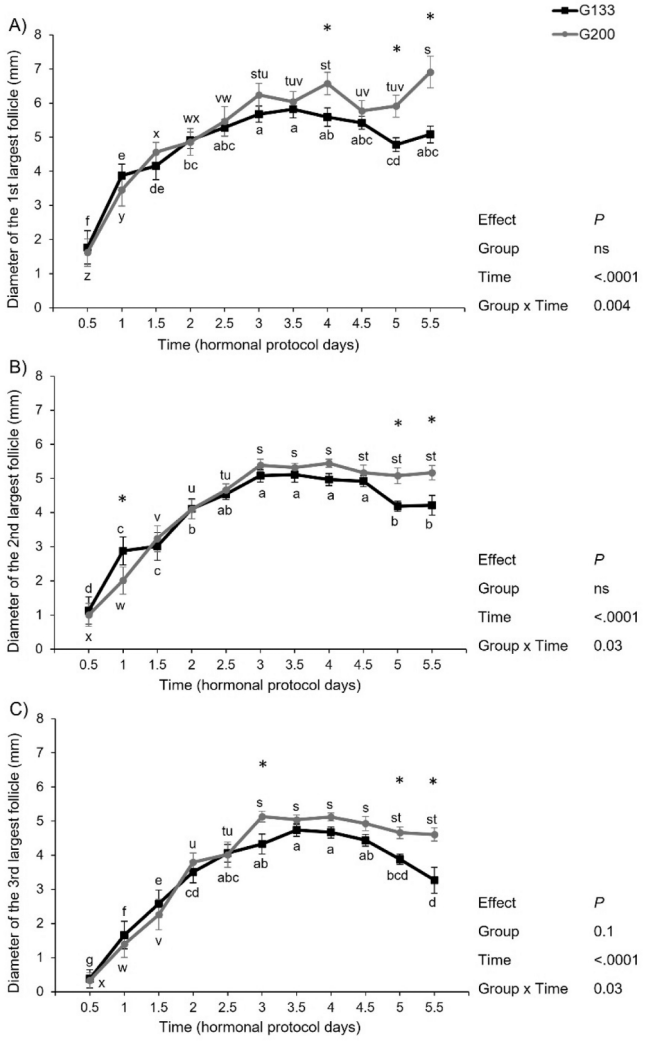

Fig. 3. Diameter of the A) first, B) second, and C) third largest follicle (mm) during the superovulatory treatment with a total dose of 133 mg (G133, -■-) or 200 mg (G200, -●-) of pFSH, based on in the Day 0 protocol in Santa Inês ewes. Follicular status was assessed by B-mode transrectal ultrasound every 12 h from the first dose of pFSH until 36 h after lecirelin administration (Day 0.5 to Day 5.5). Different lowercase letters indicate differences over time in each treatment (G133: a, b, c, d, e, f, g; G200: s, t, u, v, w, x, y, z; P < 0.05). *Indicates differences between groups at the same assessment time (P < 0.05). ns: not significant.

3.2. Estrous incidence, ovulations, embryo production, and lipid content

The treatments did not affect the percentage of animals that came into estrus, nor the interval from the removal of the P4 intravaginal device and the onset of estrus, but estrus was longer in G200 than in G133 (P=0.04) (Table 1). Treatments did not affect the proportions of ewes responsive to the treatments, ewes in which it was possible to transpose the cervix, the number of CLs, the number of anovulatory follicles per ewe, the proportion of ewes from which at least one structure was recovered, the number of recovered structures, the number of viable embryos, the proportions of blastocysts/embryos and morulaes/embryos, the viability rate, and the recovery rate. The number of freezable embryos was greater in G200 than G133 ewes (P=0.03). Treatments did not affect the lipid content (G133: 54.8 ± 6.2 *vs.* G200: 65.4 ± 4.5 arbitrary fluorescence units) nor the number of blastomeres (G133: 98.1 ± 13.8 *vs.* G200: 96.9 ± 16.9) of embryos stained with Nile Red and Hoechst, respectively (Fig. 4).

4. Discussion

In general, both doses of pFSH promoted follicular recruitment and growth, with similar and good superovulatory responses. However, although the use of 200 mg of pFSH did not affect the number of viable embryos and the proportion of blastocysts and morulaes recovered, it led to a greater production of freezable embryos. Thus, these data suggest that the 200 mg dose may have optimized the influence of FSH on oocyte developmental competence and consequently improved the quality of the embryos produced. FSH participates in the acquisition of oocyte competence during the follicular development, as it stimulates the proliferation of granulosa cells in the initial preantral phase, prevents atresia, induces the synthesis of luteinizing hormone (LH) receptors and the expression of steroid hormones (Adriaens et al., 2004; Sirard et al., 2007). Moreover, modulation by FSH concentration is essential for the *in vivo* or *in vitro* development of oocytes, and low or supraphysiological doses can interfere with the expression of transcripts related to oocytes or cumulus cells (Sánchez et al., 2010). All this, according to the present results, impact on the cryoresistance of the *in vivo* produced embryos. This implies that, at least according to the responses observed in this study, the FSH dose can be reduced to produce fresh embryos, although the final effectiveness of those embryos should be tested evaluating the results of their development in receptor ewes. On the other hand, a more detailed cost-effectiveness calculation considering the hormonal costs and the final number of high-quality embryos surviving should be considered when embryos are collected for cryopreservation. Therefore, considering these results, the treatment should be adjusted according to the aim of the final destination of embryos produced.

The amount of pFSH administered during the superovulatory treatment impacted on the follicular growth, as more medium and large follicles and a greater size of the largest follicles were observed during some periods in G200 ewes. It should be considered that on Day 2, when G200 ewes achieved a greater number of growing follicles, the females from this group had already received the equivalent of the total dose of pFSH in G133. Figueira et al. (2020) also observed a greater number of follicles starting from the second dose of pFSH, in donors who received 200 mg than in those that received 100 mg. It is, however, intriguing that although more follicles were recruited and grew more, this did not increase the number of ovulations. Thus, the limitation to further enhancement of the superovulatory response with G200 treatment is related to the maintenance of those follicles, and the proportion of large follicles from those that initially grew that finally ovulate. The longer receptiveness in G200 ewes is consistent with the presence of more large follicles, probably secreting more estradiol, but at the same time, this reinforces the idea that the greater follicular development did not increase the superovulatory response. Therefore, the step from recruitment to ovulation appears as a limiting factor for enhancing the superovulatory response with the higher FSH dose, so this should be also considered for designing future studies aiming to avoid the regression and atresia of follicles that were initially recruited by the treatment.

In this study, the FSH dose did not affect the quality of the CLs, differing from what was reported by Rodriguez et al. (2019) who

Table 1
Ovarian response and *in vivo* embryo production in Santa Inês ewes superovulated with a total dose of 133 or 200 mg of pFSH and submitted to non-surgical embryo recovery.

	G133	G200	*P*
Estrous behavior (%)	94.4 (17/18)	100.0 (18/18)	ns
Onset of estrus (hours from P4 device removal)	28.2 ± 1.7	24.0 ± 1.9	ns
Duration of estrus (h)	40.2 ± 3.6[b]	54.0 ± 4.5[a]	0.04
Cervical transposition (%)	77.8 (14/18)	78.9 (15/18)	ns
CL/ewe	8.5 ± 1.1	10.2 ± 1.1	ns
Anovulatory follicle/ewe	1.2 ± 0.3	1.4 ± 0.3	ns
Washed ewes with at least one structure recovered (%)	78.6 (11/14)	80.0 (12/15)	ns
SOV responsive ewes (%)	78.9 (15/18)	88.9 (16/18)	ns
Recovered structures	4.8 ± 1.1	7.5 ± 1.7	ns
Recovery rate (%)	47.7 ± 9.4	64.2 ± 12.6	ns
Viable embryos	3.0 ± 0.8	6.5 ± 1.6	ns
Viability rate (%)	57.8 ± 11.6	69.7 ± 9.8	ns
Freezable embryos	2.3 ± 0.7[b]	6.5 ± 1.6[a]	0.03
Blastocysts/embryos (%)	25.0 ± 12.3	70.7 ± 10.2	ns
Morulaes/embryos (%)	56.8 ± 14.2	29.3 ± 10.2	ns

Data are presented LS mean or mean ± standard error of mean. G133: 133 mg group; G200: 200 mg group; P4: progesterone; CL: corpus luteum; SOV: superovulation; ns: not significant.

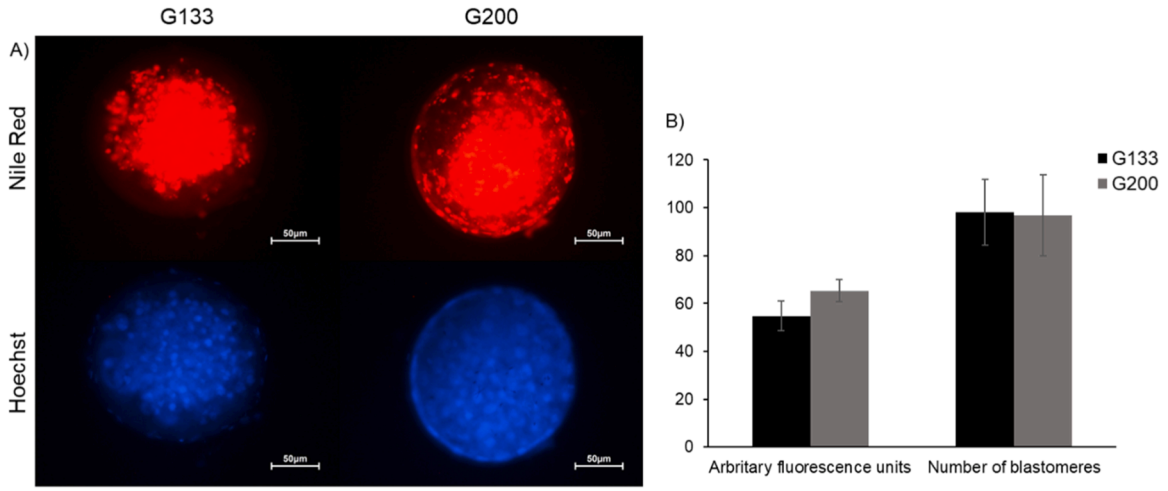

Fig. 4. A) Embryos stained with Nile Red and Hoechst evaluated under an epifluorescence microscope (200x magnification). B) Effect of superovulation treatment with a total dose of 133 mg (G133, black) or 200 mg (G200, gray) of pFSH on lipid accumulation (fluorescence emission in arbitrary fluorescence units) and number of blastomeres in *in vivo* produced embryos of Santa Inês sheep.

observed that the administration of a lower dose of pFSH reduced the incidence of premature regression of CLs. In this sense, it has been proposed that the development of a great number of big follicles might impact on the advancement of the luteolytic process. However, according to the present results the enhancement of the preovuatory follicular growth per se does not imply a reduction of CLs quality.

The FSH dose did not influence NSER and recovery rate. The average of 6.5 viable embryos collected by NSER in the present study using 200 mg of pFSH is close to the world average of 7.1 ovine embryos per flushing procedure (Viana, 2022). Performing NSER in ewes has proven to be a viable alternative to embryo collection by surgical methods, offering less risk to the health and fertility of donors, reducing the welfare concern related to invasive handlings (Fonseca et al., 2016; Santos et al., 2020; Ribeiro et al., 2023). The use of 100 mg or 200 mg of pFSH in Lacaune ewes did not change the rate of cervical transposition and uterine flushing, but the time required for cervical penetration was greater when the lowest dose was used. Also, the number of washed ewes with at least one recovered structure and embryo recovery rate was higher in the group that received 200 mg (Figueira et al., 2020). Dias et al. (2023) using another pFSH commercial preparation, found no influence of this hormone dose on NSER efficiency and recovery rate. For these authors, higher doses of pFSH may tend to facilitate NSER due to the greater number of CLs available, which would increase prostaglandin precursors and, consequently, the degree of cervical relaxation. Therefore overall, the failure of this technique seems to be more related to the physiological and anatomical characteristics of the breed rather than to the superovulatory treatment.

The pFSH dose did not interfere with the accumulation of intracellular lipid droplets, so it is unlikely that this is a key mechanism for the differences in freezable embryos observed in the present study. Although not fully understood, FSH can interfere with gonadal and extragonadal lipid metabolism (Cui et al., 2012; Liu et al., 2015; Kumar, 2018). Wang et al. (2015) reported that SOV affects lipid metabolism in the ovary and its effect was sustained until the two-cell embryonic stage in mice. These authors found that the use of gonadotropins increased the ovarian cholesterol content and altered the expression of genes related to steroidogenesis. Additionally, there was a dose-dependent effect on the accumulation of lipids and the synthesis and composition of fatty acids in the embryos produced. The excessive formation of intracellular lipid droplets can compromise embryonic development competence and impair cryopreservation, increasing sensitivity to cryoinjuries during this process (Prates et al., 2014; Romão et al., 2016; Amstislavsky et al., 2019). Performing further analysis on embryos and post-transfer offspring in studies evaluating different superovulation protocols in sheep could provide even more information on the potential impact of gonadotropin dose on the relationship between lipid content and embryonic quality.

5. Conclusion

The two doses of pFSH used (133 mg or 200 mg) promoted an efficient superovulatory response in Santa Inês ewes and did not affect embryonic lipid accumulation. The recovery of viable embryos was similar, but the greater FSH dose enhanced the follicle recruitment and allowed achieving a greater number of freezable embryos, suggesting that this dose has the potential use to increase the superovulatory results.

CRediT authorship contribution statement

Rodolfo Ungerfeld: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **Jeferson Ferreira da Fonseca:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Mario Felipe Alvarez**

Balaro: Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Camila Correa Roza Laeber:** Investigation, Data curation. **Joanna Maria Gonçalves Souza-Fabjan:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Ribrio Ivan Tavares Pereira Batista:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Pedro Henrique Nicolau Pinto:** Data curation, Investigation, Writing – review & editing. **Ana Clara Sarzedas Ribeiro:** Investigation, Formal analysis, Conceptualization, Data curation, Methodology, Writing – original draft, Writing – review & editing. **Nadiala Porto Ramalho:** Investigation, Data curation. **Juliana Dantas Rodrigues Santos:** Data curation, Investigation, Writing – review & editing. **Felipe Zandonadi Brandão:** Conceptualization, Data curation, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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