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The coasting time affects the quality of cumulus-oocyte complexes in superstimulated ewes

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ABSTRACT

We hypothesized that the coasting time may be beneficial to the quality of cumulus-oocyte complexes recovered from live ewes, as reported in cattle. The present study assessed the effect of coasting times on the quantity and quality of cumulus-oocyte complexes (COCs) in sheep. All ewes were subjected to the "Day 0 protocol", followed by an ovarian stimulation (80 mg of pFSH in three decreasing doses), varying only the coasting time [12 (G12), 36 (G36), or 60 h (G60]. In Experiment 1, data regarding follicular population was assessed. In Experiment 2, the COC quality was checked by their morphology, brilliant cresyl blue (BCB) test, evaluation of chromatin condensation pattern, and oocyte diameter. In Experiment 3, genes related to oocyte developmental competence were evaluated in BCB + COCs. The oocytes in the G60 group had more ($P < 0.05$) large follicles than the other groups and oocytes with a greater diameter than the G12. Oocyte morphology was similar ($P > 0.05$) among groups, as well as the BCB + COCs quantity. The G60-oocytes presented a better ($P < 0.05$) configuration of chromatin condensation compared with the other groups and a greater ($P < 0.05$) gene expression of *BMP15, MATER, ZAR1*, and *PTGS2* compared with G12, and *PTGS2* and *HAS2* compared with G36 group. In conclusion, 60 h of coasting time positively affects the quality of COCs recovered after subjecting ewes to the "Day 0 protocol" and ovarian superstimulation. Implementing the appropriate coasting time to a given protocol can positively impact the *in vitro* embryo production outcomes in sheep.

1. Introduction

The recovery of good-quality cumulus-oocyte complexes (COCs) is essential to any laboratory involved in *in vitro* embryo production (IVEP), as well as in cloning or transgenesis. Overall, the high variability in the number and quality of COCs retrieved is one of the main bottlenecks impairing the worldwide propagation of IVEP in small ruminants (for a review, see Ref. [1]). In such species, laparoscopic ovum pick-up (LOPU) is the method of choice for obtaining oocytes from live females. It is a safe technique, that allows successive collections, although it demands hormonal protocols to improve the number of COCs recovered and their developmental capacity, with results relying on the FSH dose and regimen of administration adopted [2,3]. In order to optimize the ovarian superstimulation protocol in sheep, our group applied the "Day 0 protocol" (follicular wave synchronization) and evaluated four different treatments, varying only the pFSH dose and application regimen [4]. As a result, the use of the 80 mg pFSH dose provided the highest COCs quality, with an adequate gene expression pattern when applied in multiple (three) doses. Subsequently, it was demonstrated that the use of exogenous progesterone (P4) during the ovarian superstimulation protocol, compared to its analogue (medroxyprogesterone acetate), had positive effects on gene expression and oocyte competence [5]. Importantly to note is that a standard 12 h-interval from the last pFSH dose to the LOPU procedure (coasting time) was applied in both studies.

In cattle, the coasting time has been studied for about 25 years [6–8] as an alternative to improve IVEP results. In 1997, a group of researchers observed that the interval from FSH administration to

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slaughter, as well as from slaughter to oocyte retrieval, would impact the number of competent bovine COCs [9]. These authors speculated that the LH window – the period between luteolysis and the next LH surge – was shortened in superstimulated animals [10,11], which could affect the oocyte developmental competence. Later, it was reported that defining a "competence window" by adjusting the coasting time was extremely important for enhancing the oocyte competence and IVEP rates in cattle [7]. Even though the importance of defining the ideal coasting time in bovine species has been evident for many years, the literature is still incipient in sheep.

For reaching its developmental competence, the oocyte needs to accumulate inactive maternal transcripts, which will control all processes from follicle removal up to embryonic genome activation (for reviews, see Refs. [12,13]). Genes such as *MATER*, *ZAR1*, *GDF9*, *BMP15*, *HAS2*, and *PTGS2*, are indicators of oocyte competence and their expression analyses can better predict the oocyte quality [4,14,15]. Aiming for such prediction, the morphological evaluation in degrees is widely used worldwide, however, this classification is considered subjective and not a good quality predictor. The brilliant cresyl blue (BCB) test is an alternative predictor of oocyte quality that promotes the selection of a homogeneous and competent pool of oocytes in the final folliculogenesis period, when the follicle reaches its growth peak and there is full oocyte competence acquisition [2,16,17]. Another interesting possible predictor of oocyte quality is the chromatin condensation pattern of the germinal vesicle, which is remodeled during oocyte growth and is related to its development, seeming to coordinate the control of global transcription and define the developmental potential of a germinative cell [18]. Moreover, there is also a correlation between the oocyte quality and its size, as well as the follicle size, with the oocyte competence increases according to the follicle size [19].

Considering the above, we hypothesized that the coasting time may be beneficial, improving the COC quality in sheep, as in cattle [7]. Therefore, the present study aimed to evaluate in ewes the effect of different coasting times on: (1) follicular dynamics, follicle size, and COCs quantity; (2) COC or oocyte quality assessed by their morphology, BCB test, germinal vesicle chromatin condensation pattern evaluation, and oocyte diameter; and (3) gene expression analyses of COC competence markers.

2. Material and methods

2.1. Ethics, location, and conditions

The procedures were approved by the Ethics Committee for Animal Use of the Universidade Federal Fluminense (Protocol #7226291020/2020). The study was conducted in June of 2021 (breeding season) at the Unidade de Pesquisa Experimental em Caprinos e Ovinos (UNIPECO) of Universidade Federal Fluminense, in Cachoeiras de Macacu, Rio de Janeiro, Brazil (22° S, 42° W). Thirty-six adult Santa Ines ewes were chosen and considered clinically healthy after clinical, gynecological, and ultrasound examination. They had a mean bodyweight of 46.0 ± 1.0 kg and a mean body condition score of 3.96 ± 0.11. They were housed and received chopped elephant grass (*Pennisetum purpureum*) and 200 g concentrate per animal twice daily, and water and mineralized salt were provided *ad libitum*. The animals were kept in confinement under sanitary, environmental, and nutritional control throughout the study.

2.2. Experimental design

The experimental design is illustrated in Fig. 1. Thirty-six Santa Ines ewes underwent ovarian stimulation after "Day 0 protocol" using three different protocols, varying only with the coasting times used (12, 36, or 60 h). The best protocol of Bragança et al. [5]

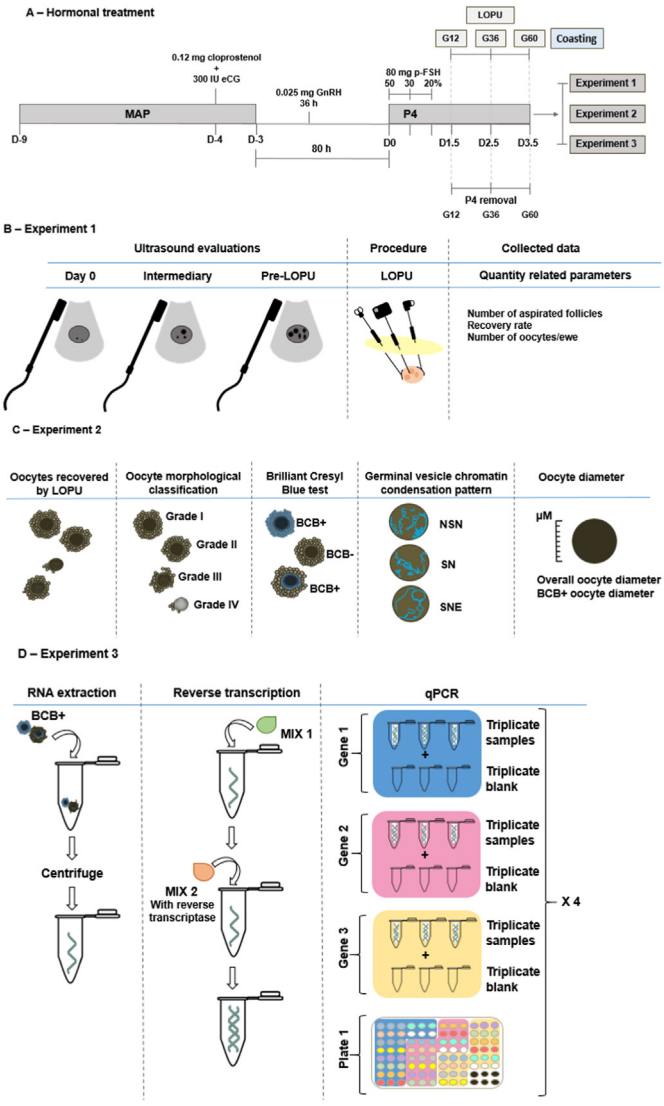

Fig. 1. A - Experimental design: Thirty-six Santa Inês ewes were subjected to the "Day 0 protocol" for synchronization of follicular wave emergence, followed by ovarian superstimulation with three decreasing doses of 80 mg of pFSH (50%, 30%, and 20%). Ewes were allocated into one of three different groups (G12, G36, and G60), differing only in coasting times (12, 36, or 60 h, respectively). The same protocol and treatment groups were used throughout experiments. B – Experiment 1: Ewes were subjected to LOPU and parameters such as follicular population and cumulus oocyte complexes (COCs) recovery and viability rates were assessed. C – Experiment 2: After LOPU, COCs were morphologically graded as described in Souza-Fabjan et al. (2016), stained with Brilliant Cresyl Blue (BCB test), and classified as BCB+ (blue cytoplasm) and BCB- (colorless cytoplasm). Then, they were denuded and stained with Hoechst to assess chromatin configuration patterns and measured to verify their diameter. D – Experiment 3: BCB + COCs were snap-frozen in DNA and RNA-se free for gene expression analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

– 80 mg of pFSH in multiple decreasing doses, with 12 h of coasting – was considered as the control treatment. In Experiment 1, transrectal ultrasonography was performed to count and measure the ovarian antral follicles, and some parameters such as follicle size, number of oocytes/ewe, recovery, and viability rate were recorded. In Experiment 2, the recovered COCs were graded morphologically according to Souza-Fabjan et al. [20] and tested using BCB. The BCB-positive (BCB+) and negative (BCB-) COCs were used for both the germinal vesicle chromatin condensation pattern analysis and oocyte diameter. In Experiment 3, BCB + COCs were submitted for gene expression analysis.

2.3. Hormonal stimulation

Estrus synchronization was performed with intravaginal sponges containing 60 mg medroxyprogesterone acetate (Progespon®; Schering Plough Animal Health) for 6 days. One day before sponge removal, 300 IU eCG (Novormon 5000®; MSD Animal Health) and 0.12 mg cloprostenol sodium (Estron®; Tecnopec), as well as 0.025 mg lecirelin (Gestran Plus®; Tecnopec) 36 h after progestogen removal [21], were administered intramuscularly (i.m.). After 80 h of sponge removal, a P4 intravaginal device (Primer®, União Química Farmacêutica Nacional) was inserted, ovarian stimulation was initiated (considered Day 0) by the administration of 80 mg pFSH (Folltropin-V®, Bioniche Animal Health) divided into three decreasing doses (40, 24 and 16 mg, respectively) every 12 h [5]. Three coasting times were defined, i.e., LOPU occurred at three different time intervals from the last pFSH administration: G12 (control group) − 12 h; G36 − 36 h; and G60 − 60 h.

2.4. Experiment 1

2.4.1. Ovarian assessment by ultrasonography

The follicular population was assessed by transrectal ultrasonography immediately at the beginning of ovarian stimulation (Day 0), at an intermediary time [at 12 h (G12) or 24 h (G36 and G60)], and immediately before LOPU. Ovulatory parameters such as the number, position, and diameter of the ovarian follicles were recorded. Follicles were classified [4] according to their size as small (diameter <3.0 mm), medium (diameter 3.1–5.0 mm), or large (diameter >5.0 mm). Evaluations were performed using an ultrasound (MindRay Z5®, Shenzhen, China) equipped with a 7.5-MHz linear transducer in B-mode.

2.4.2. COC recovery by LOPU

Ewes received only 50% of food 48 h before laparoscopy. One day before LOPU, they were deprived of 100% food, and 12 h before the procedure water was also withdrawn. Pre-anesthesia, anesthesia, and LOPU procedures were performed as described by Bragança et al. [4]. Briefly, 0.2 mg kg^{-1} (i.v.) diazepam (Diazepam®; Teuto, Anápolis, Brazil), 0.1 mg kg^{-1} (i.v.) acepromazine (Acepran®; Vetnil, Brazil) and 0.4 mg kg^{-1} (i.m.) morphine (Dolo Moff, Brazil) were administered. Anesthesia was induced with 1% propofol (Profolen®; Balusiegel, Brazil) using the dose-response method and maintained with isoflurane (Forane®; Abbott Laboratories, USA) via endotracheal intubation. A 2% lidocaine (Lidovet®; Bravet, Brazil) was injected for local anesthesia.

Visible follicles (G12: n = 132; G36: n = 125; G60: n = 120) were aspirated at LOPU using a 20-G needle with a bevel connected to a cannula, connected to a silicone cork (WTA, Brazil) inserted in a 50 mL tube. The aspiration pressure was regulated (36–40 mmHg) by a vacuum pump (WTA). The aspiration medium was a TCM 199 (M2520; Sigma-Aldrich) supplemented with 20 IU/mL heparin (Calbiochem 375095), 0.2 mM pyruvate (P4562; Sigma), 100 IU penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin (A5955; Sigma), and 1% bovine serum albumin (BSA; A9647; Sigma).

2.5. Experiment 2

2.5.1. COCs grading and BCB test

The recovered COCs (G12: n = 70; G36: n = 56; G60: n = 63) were evaluated under a stereomicroscope and graded based on morphology assessment according to Souza-Fabjan et al. [20]. For inferring the developmental competence, viable COCs (Grades I–III) from each treatment (G12: n = 68; G36: n = 52; G60: n = 58) were washed in Dulbecco's modified phosphate-buffered saline supplemented with 1% BSA (A9647, Sigma), followed by incubation in 26 mM BCB (B5388, Sigma) diluted in DMPBS with 1% BSA (Forma Series 3 Water Jacket CO_2 Incubator − Model 4100 Series) for 60 min at 38.5 °C. Subsequently, the COCs were washed twice in DMBPS with 1% BSA and classified according to oocyte cytoplasm staining as BCB+ (blue cytoplasm) or BCB- (colorless cytoplasm).

2.5.2. Germinal vesicle chromatin configuration analysis and oocyte diameter

After the BCB test, COCs were denuded by vortexing, recovered, fixed in 4% paraformaldehyde, and stored until analysis. To evaluate the germinal vesicle chromatin condensation pattern, the oocytes (G12: n = 40; G36: n = 31; G60: n = 30) were individually stained with 1 μg/mL of Hoechst 33342 in the dark, mounted on slides with coverslips, and analyzed under epifluorescence microscopy (Nikon Eclipse Ci − Nikon Corporation − JP). The germinal vesicle chromatin condensation pattern was classified as: diffuse chromatin in the whole nuclear area (nonsurrounded nucleolus, NSN); condensed chromatin surrounding the nucleolus (surrounded nucleolus, SN); or condensed chromatin near the nucleolus and the nuclear envelope (SNE) [18]. Photos were recorded, and the denuded oocytes were individually measured by a pre-calibrated software (ImageJ, National Institutes of Health, Bethesda, MA, USA) to determine the diameter (without zona pellucida). Two measurements were taken (vertical and horizontal), and the diameter was considered as their mean.

2.6. Experiment 3

2.6.1. Gene expression analysis

After the BCB test, three pools of five BCB + COCs from different females of each treatment (G12, G36 and G60) were frozen in DNAse and RNAse-free cryotubes with a minimal amount of medium and stored in a liquid nitrogen bank for later gene expression analysis. To assess the abundance of mRNA from six genes encoding protein markers of oocyte competence (*MATER*, *ZAR1*, *BMP15*, *GDF9*, *HAS2*, and *PTGS2*) and two apoptosis-associated genes expressed in both cumulus cells and oocytes (*BAX* and *BCL2*), two reference genes were used as calibrators to compare the parameters of physiological molecular status with each treatment (*GAPDH* and *H2AFZ*) (Table 1). Total RNA extraction was performed using RNeasyMicro Kit (Qiagen Inc., Valencia, EUA) according to the manufacturer's instructions. RNAase free water (14 μL) was added, and the RNA quantification of each pool was performed using 1 μL of sample on a ND-100 spectrophotometer (NanoDrop™ Lite Spectrophotometer), which resulted in a mean (ng) total RNA/COCs of 10.8 ± 3.8; 4.6 ± 1.3 and 9.4 ± 2.0 for the G12, G36, and G60 groups, respectively. The SuperScript IV first-strand synthesis Supermix (Invitrogen, Carlsbad, CA, USA) was used for reverse transcription, and the same RNA concentration was used in all samples. A mixing of oligo (dT) 20 primers, dNTP mixture, Superscript IV-RT, RNase OUT, $MgCl_2$, RT buffer, and RNA sample in a final volume of 20 μL, was prepared to do the reverse transcription reaction. The mixtures were incubated at 65 °C for 10 min, followed by 50 °C for 10 min and 80 °C for 10 min. The relative quantification was done using a real-time polymerase chain reaction (QuantStudio™ 3 Real-Time PCR System) in triplicates. Reactions (20 μL total volume) were made with a mixture of SYBR green kit (10 μL; Power SYBR Green, Applied Biosystems), 0.1 μM primers (described in Table 1), nuclease-free water, and reverse-transcribed cDNA (0.5 μL). Negative controls were similarly run with each group of samples containing the RT-qPCR reaction mixture without nucleic acids. Template cDNAs were denatured at 95 °C for 10 min, the gene amplification was amplified by 40 cycles of thermal cycling

Table 1
Sequence of the primers used in the gene expression analysis of brilliant cresyl blue positive cumulus-oocyte complexes (BCB + COCs) recovered by laparoscopy from ewes subjected to hormonal superstimulation protocol with three different coasting times (12, 36, or 60 h).

Gene	Primer sequence (5′-3′)	Size(bp)	Reference
BAX	Forward: CCT GGG ATC TTG AAA CTC TCC TT Reverse: CTG GC CAG GCT GAA ATC AAA A	566	[39]
BCL2	Forward: GCC GAG TGA GCA GGA AGA C Reverse: GTT AGC CAG TGC TTG CTG AGA	214	[39]
BMP15	Forward: GGG TTC TAC GAC TCC GCT TC Reverse: GGT TAC TTT CAG GCC CAT CAT	273	[14]
GAPDH	Forward: GGG AAA TCG TGC GTG ACA TTA AG Reverse: TGT GTT GGC GTA AGG TCT TTG	273	[40]
GDF9	Forward: CAG ACG CCA CCT CTA CAA CA Reverse: CAG GAA AGG GAA AAG AAA TGG	198	[14]
H2AFZ	Forward: GTC GTG GCA AGC AAG GAG Reverse: GAT CTC GGC CGT TAG GTA CTC	182	[41]
HAS2	Forward: CCT CAT CCA AAG CCT G Reverse: ACA TTT CCG CAA ATA GTC TG	138	[15]
MATER	Forward: CAG CCT CCA GGA GTT CTT TG Reverse: GAC AGC CTA GGA GGG TTT CC	212	[14]
PTGS2	Forward: AGG AGG TCT TTG GTC TGG TG Reverse: TCT GGA ACA ACT GCT CAT CG	126	[15]
ZAR1	Forward: CAC TGC AAG GAC TGC AAT ATC Reverse: CAG GTG ATA TCC TCC ACT C	137	[14]

BAX: BCL2 associated protein X; BCL2: B-cell lymphoma protein 2; BMP15: bone morphogenetic protein 15; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GDF9: growth differentiation factor 9; H2AFZ: H2A histone family, member Z; HAS2: hyaluroran synthase 2; MATER: maternal antigen that embryo requires; PTGS2: prostaglandin-endoperoxide synthase 2; ZAR1: zygote arrest 1.

programmed at 95 °C for 15 s, 60 °C for 15 s, and 60 °C for 30 s. Fluorescence data were acquired during the extension steps. After each RT-qPCR run, a melting curve analysis was performed to confirm that a single specific product was generated. Primer efficiency was calculated using LinRegPCR software [22] for each reaction. Primer efficiency standard was 1.91; 1.92; 1.94; 1.93; 1.91; 1.96; 1.91; 1.95; 1.93 and 1.98 to *MATER*, *ZAR1*, *BMP15*, *GDF9*, *HAS2*, *PTGS2, BAX*, *BCL2, GAPDH,* and *H2AFZ*.

2.7. Statistical analysis

Statistical analyses were performed by generalized linear models using SAS software version 9.3 (SAS Institute Inc., Cary, NC). Continuous variables were evaluated for normality using PROC UNIVARIATE. Dependent variables with normal (parametric) distribution were analyzed using PROC GLM, and Tukey test to perform multiple comparisons. Non-parametric data with POISSON or GAMMA distribution were analyzed with PROC GLIMMIX and log-link function. The number of follicles at Day 0 and at an intermediary time [at 12 h (G12) or 24 h (G36 and G60)] were evaluated as repeated measures. Binomial variables were expressed as a percentage and analyzed using PROC GLIMMIX with logit-link function. Variables were expressed as means ± s.e.m. and the statistical significance value was 5%, or $P < 0.05$. In the statistical analysis of gene expression, the stability of the *GAPDH* and *H2AFZ* genes were calculated using BestKeeper − Excel. To perform the relative quantification, the comparative method of Ct ($2^{-\Delta\Delta Ct}$) was used, with the REST 2008 software [23] and the efficiencies obtained with the LinRegPCR software. The expression of the target genes was normalized using the geometric mean of the values of the reference genes.

3. Results

3.1. Experiment 1

The first two ultrasound evaluations performed before the treatment was applied, i.e., at the beginning of ovarian stimulation (Day 0) and at an intermediary time [at 12 h (G12) or 24 h (G36 and G60)], indicated that there were no differences ($P > 0.05$) among treatment groups regarding the number of follicles in each category (small, medium, and large). In the pre-LOPU evaluation, the number of small follicles in G12 and G36 was higher ($P < 0.05$) when compared to G60, while the number of medium follicles did not differ ($P > 0.05$) among groups. The number of large follicles was greater ($P < 0.05$) in G60 compared to the other coasting times. Data regarding the follicular population are presented in Fig. 2. The total number of aspirated follicles as well as the quantitative parameters of COC recovery did not differ ($P > 0.05$) among groups (Table 2). It was not possible to proceed with follicle aspiration of one sheep from G12 group due to problems in the LOPU system.

3.2. Experiment 2

Qualitative aspects of COCs produced from the three different treatments are shown in Table 3. There was no difference ($P > 0.05$) among treatments in any parameter related to COCs morphology. Moreover, the oocytes derived from the G60 group had a greater diameter ($P < 0.05$) compared to G12 and both groups were similar ($P > 0.05$) to the intermediary time, G36. Although the SN germinal vesicle chromatin configuration pattern did not differ ($P > 0.05$) among groups, the frequency of the NSN and SNE were lower and higher ($P < 0.05$), respectively, in the G60 group.

3.3. Experiment 3

The overall gene expression analysis is shown in Fig. 3. When analyzing G12 vs G36, *GDF9*, *BMP15*, *MATER*, and *ZAR1* genes were upregulated in G36, while *BCL2*, *BAX*, and *PTGS2* genes did not differ among the aforementioned groups. The *HAS2* gene was downregulated in G36 in relation to G12. Comparing G12 vs G60, the *BMP15*, *MATER*, *ZAR1*, *PTGS2*, and *BCL2* genes were upregulated in G60, while the other genes did not differ. When G36 vs G60 was compared, the *GDF9*, *BMP15*, *MATER*, *ZAR1*, and *BAX* genes showed similar expression levels, while *PTGS2*, *HAS2*, and *BCL2* were upregulated in G60. The *BAX-BCL2* ratio was less than one in all groups (0.9; 0.8; and 0.7 in G12, G36, and G60, respectively).

4. Discussion

The main objective of the present study was to evaluate the effect that different coasting times (12, 36, or 60 h) would have on the quantity and quality of COCs in sheep. We hypothesized that the use of different coasting times could result in different COC quality in sheep, as reported in cattle [7]. From the data obtained in this study, we were able to obtain four main conclusions. Firstly, little variation was found in LOPU-related parameters and COC morphological evaluation under different coasting times. Secondly, the number and the percentage of BCB + COCs were similar among the different treatments, although a tendency favoring the prolonged coasting (60 h) was observed in the percentage of BCB + COCs. Thirdly, regarding the chromatin condensation pattern analysis, the SNE chromatin condensation pattern was more common in the G60 group than in the others, indicating that longer coasting is beneficial to the oocyte. Finally, the results of gene expression indicate that more advanced coasting times (36 and 60 h) allow the oocyte to accumulate more transcripts and, consequently, have a better developmental capacity. These conclusions demonstrate that, in sheep, a prolonged coasting time (60 h) provides better quality oocytes, suggesting a greater competence for blastocyst development.

The superstimulatory response, mean recovery rate, number of

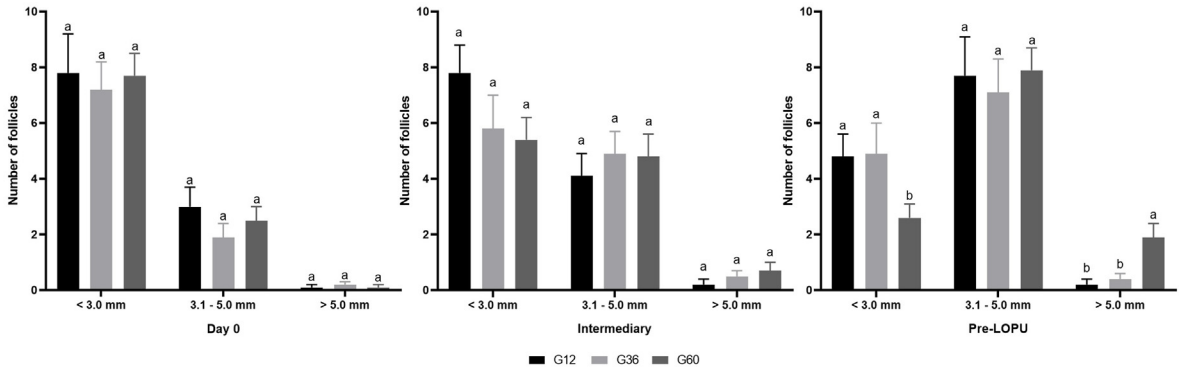

Fig. 2. Follicular population at the beginning of ovarian stimulation (Day 0), at an intermediary time [at 12 h (G12) or 24 h (G36 and G60)], and immediately before laparoscopic ovum pick up (LOPU) – Experiment 1.
Unless indicate otherwise, data are the mean ± s.e.m. (n = 12 ewes per treatment). Within rows, values with different uppercase superscript letters differ significantly ($P < 0.05$).

oocytes/ewe, number of good-quality COCs/ewe, and number of viable COCs/ewe did not differ significantly among groups. Our data are similar to those obtained by Bragança et al. [4,5], who worked with the same superstimulation protocol and breed as our control group. In the current study, the recovery rate, and percentage of good-quality oocytes were similar to those obtained in Merino ewes [3], as well as the number of GI/GII COCs and the number of recovered COCs in the Merino breed [24]. Based on these data, it is possible to state that the superstimulation protocol used in our study was able to generate an efficient ovarian response, allowing the collection of COCs with good morphological quality and of high capacity for development, as observed in the BCB test.

In Experiment 1, we can infer from the analysis of the follicular population in each diameter class that the "Day 0 protocol" allowed a standardized first wave of follicular development for all groups. This was the expected growth of follicles, in which they evolved from a small to a large size. Such progression throughout the superstimulation protocol, observed at the ultrasound exams, indicates that the protocol was well conducted and effective in stimulating large numbers of oocytes from a synchronized emergence wave [21]. The absence of any difference among the treatments in the total number of follicles agrees with previous findings in cattle and buffalo [7,25], showing that the coasting time apparently has no role on the number of follicles available for harvesting and, thus, the COC quantity. Conversely, in the present study, longer coasting (60 h) resulted in significantly more large follicles compared to shorter coasting times, similar to observations in cattle [7], but in contrast to results from buffalo [25].

The increase in follicle size in G60 resulted in larger COCs compared to the other groups. It is known that oocytes that have larger sizes also have a greater capacity to store maternal transcripts that will be used in their initial zygotic phase [2]. Therefore, we can deduce that COCs recovered after 60 h of coasting, derived from larger follicles and presenting a larger diameter, presumably have a greater developmental capacity [26] than COC obtained after shorter coasting times. In addition, the tendency of G60 to have a higher percentage of BCB + COCs, and the significant higher pattern of SNE chromatin condensation – considered the most advanced chromatin condensation pattern until ovulation [18] – also corroborate with the hypothesis that a longer coasting time is beneficial to the growth and competence development of the oocyte. Interestingly, detection of conclusive results was impossible with only the morphological evaluation of the COCs, which, as already known, is not a good criterion for quality evaluation, since grade I oocytes are at an early stage of development [27]. However, complementary analyses such as the BCB test, the germinal vesicle chromatin condensation pattern evaluation, and the expression of specific genes of oocyte competence provide more information about the predictability of oocyte competence.

Regarding the gene expression, the *GDF9* and *BMP15* factors have fundamental roles in the regulation of follicular development and oocyte maturation [15]. Such factors, secreted by the oocyte, should not be considered independently, but as an important functional unit of signaling in ruminants [28]. In the present study, *GDF9* was expressed lower in G12 compared to G36, but not in G60. The increase in their mRNA levels in humans is correlated with oocyte maturation, oocyte competence, and embryo quality [29]. Maside et al. [15] pointed out that the levels of *GDF9* and *BMP15* increase along with the acquisition of oocyte quality in some species, but in ovine, the expression of *GDF9* mRNAs was lower in oocytes that had a greater capacity for embryonic development. In the present study, although not significant, *GDF9* was downregulated in G60 compared to G36, in agreement with Maside et al. [15]. Whether or not *BMP15* acts synergistically with *GDF9*, the former is responsible for the proliferation of granulosa cells and their response to FSH, modulating follicular maturation and growth [30]. The *BMP15* gene behaved differently from *GDF9*, being upregulated in both G60 and G36, in comparison with G12, but not differing from each other. Thus, as the coasting time increased, the expression of this gene also increased, but from 36 h onwards it reached the peak of its expression, and remained similarly upregulated at G60.

Table 2
Effect of different coasting times (12, 36, or 60 h) on the quantity of cumulus-oocyte complexes (COCs) recovered by laparoscopy in superstimulated ewes – Experiment 1 (mean ± s.e.m.).

Parameters	G12 (n = 11)	G36 (n = 12)	G60 (n = 12)	Overall	P-value
Number of aspirated follicles	12.0 ± 1.3	10.4 ± 1.0	10.0 ± 1.3	10.8 ± 0.7	0.49
Recovery rate (%)	53.8 ± 5.7	46.0 ± 3.9	51.3 ± 5.3	50.1 ± 2.9	0.36
Number of oocytes/ewe	6.4 ± 0.9	4.7 ± 0.5	5.3 ± 0.9	5.4 ± 0.5	0.22

Unless indicate otherwise, data are the mean ± s.e.m. G12, 12 h of coasting; G36, 36 h of coasting; G60, 60 h of coasting.

Table 3
Effect of different coasting times (12, 36, or 60 h) on the quality of cumulus-oocyte complexes (COCs) recovered by laparoscopy in superstimulated ewes – Experiment 2 (mean ± s.e.m.).

Quality parameters	G12 (n = 11)	G36 (n = 12)	G60 (n = 12)	Overall	P-value
Number of viable COCs/ewe	6.2 ± 1.0	4.3 ± 0.5	4.8 ± 1.0	5.1 ± 0.5	0.15
Viability rate (%)	97.1 ± 2.4	92.9 ± 3.3	92.1 ± 5.6	94.2 ± 2.3	0.44
Number of good-quality COCs/ewe	4.6 ± 0.5	2.9 ± 0.5	3.0 ± 0.9	3.5 ± 0.4	0.06
Good-quality COCs rate (%)	72.9 ± 6.9	62.5 ± 9.9	57.1 ± 10.6	64.6 ± 5.5	0.31
Nb of BCB + COC/ewe	5.1 ± 1.0	3.1 ± 0.4	4.3 ± 0.8	4.1 ± 0.5	0.28
BCB + rate (%)	82.4 ± 1.0	71.2 ± 0.4	89.7 ± 0.8	81.5 ± 0.5	0.07
Oocyte diameter (μm)	114.6^{b} ± 2.8	$118.5^{a,b}$ ± 3.0	123.1^{a} ± 2.6	118.3 ± 0.9	<0.01
BCB + oocyte diameter (μm)	115.1^{b} ± 1.6	$118.7^{a,b}$ ± 1.5	123.3^{a} ± 1.3	118.9 ± 1.0	<0.01
NSN chromatin pattern (%)	95.0^{a} ± 3.4	80.7^{a} ± 7.0	43.3^{b} ± 9.0	75.2 ± 0.18	<0.01
SN chromatin pattern (%)	2.5 ± 2.4	6.5 ± 4.4	6.6 ± 4.6	5.0 ± 0.11	0.68
SNE chromatin pattern (%)	2.5^{c} ± 2.4	12.9^{b} ± 6.0	50.0^{a} ± 9.1	19.8 ± 0.15	<0.01

Unless indicate otherwise, data are the mean ± s.e.m. (n = 12 ewes per treatment). Within rows, values with different uppercase superscript letters differ significantly ($P < 0.05$). Good-quality COCs were considered those of grades I/II, whereas viable COCs were considered those of grades I-III. BCB+ was the COC with blue cytoplasm and the BCB- was the COC with colorless cytoplasm. G12, 12 h of coasting; G36, 36 h of coasting; G60, 60 h of coasting.

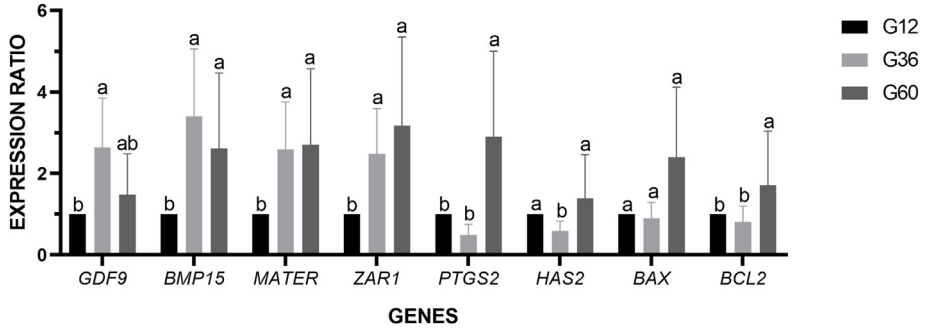

Fig. 3. Gene expression analysis of brilliant cresyl blue positive (BCB+) cumulus-oocyte complexes (COCs) derived from hormonally superstimulated ewes, after receiving different coasting times (12, 36, or 60 h) – Experiment 3. Data are show as means ± s.e.m.
The reference genes were *GAPDH* and *H2AFZ*. *BAX*: BCL2 associated protein X; *BCL2*: B-cell lymphoma protein 2; *BMP15*: bone morphogenetic protein 15; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; *GDF9*: growth differentiation factor 9; *H2AFZ*: H2A histone family, member Z; *HAS2*: hyaluroran synthase 2; *MATER*: maternal antigen that embryo requires; *PTGS2*: prostaglandin-endoperoxide synthase 2; *ZAR1*: zygote arrest 1. Within rows, values with different uppercase superscript letters differ significantly ($P < 0.05$).

The *MATER* and *ZAR1* genes are known to have a marked decrease in their expression patterns from activation of the embryonic genome, with *MATER* being undetectable at the 16-cell stage [14]. They have similar expression patterns and are required in a development period prior to zygotic genome activation. When *MATER* and *ZAR1* mRNA transcripts are highly expressed, as in groups G36 and G60 in relation to G12, they serve to predict the higher development of oocyte competence [4].

The *PTGS2* expression in COCs is related to the developmental capacity of the oocyte and embryo [31]. The high mRNA levels of *PTGS2* at G60 in this study, as well as for *HAS2*, may be correlated with the acquisition of oocyte competence. These genes have been associated with some species, such as bovine [32], porcine [33], and humans [34]. In this study, *PTGS2* was more highly expressed in G60 in relation to the other groups, while *HAS2* was more highly expressed in G60 when compared to G36 but did not significantly differ in relation to G12. *HAS2*, which participates in the final oocyte maturation [35], has its expression enhanced in humans [34] and sheep [36], and this fact was associated with a better quality of both oocyte and embryonic development. It was also highly expressed in sheep large oocytes, able to produce blastocysts, i.e., of good quality [15].

As already known, *BCL2* acts as an anti-apoptotic gene, i.e., it promotes cell survival. Meanwhile, *BAX* is a factor associated with *BCL2* that acts by promoting apoptosis, i.e., cell death [36,37]. Such factors participate in the mitochondria-dependent apoptotic pathway [15] and need to be expressed in the cell in an equilibrium situation, in equal proportion to each other, so that *BCL2* interacts with *BAX* and neutralizes its activity. If this does not occur and the proportion of *BAX* is greater than that of *BCL2*, cytochrome C is released into the cytoplasm and, consequently, caspases are activated, which indicates that the cell is initiating the cell death process. Therefore, the expression of these genes is related and can be used to predict oocyte competence [38]. The results of the present study show that the *BAX* and *BCL2* genes maintain the proportion between them (less than one) in all groups, despite *BCL2* being significantly upregulated in G60, with no evidence of cellular apoptosis in any of the groups.

Overall, our results indicate that the G60 group resulted in the formation of larger follicles, had a tendency to have more BCB + COCs, the oocytes were larger in diameter (compared with G12) and had significantly higher SNE patterns (germinal vesicle), and COCs had an enhanced pattern of expression of important gene markers of oocyte developmental competence. Altogether, evidence indicates that coasting affects oocyte quality and, possibly, the G60 group has the most competent oocytes. This will probably affect subsequent embryo quality, allowing the application of this tool to further improve reproductive biotechnologies such as IVEP, cloning, or transgenesis, which all require good-quality COCs.

5. Conclusions

In conclusion, under the conditions of the present study, the quantity and COCs grade morphology showed little effect of different coasting times. However, the G60 group had higher oocyte diameter, enhanced chromatin condensation pattern, and a greater capacity to accumulate transcripts, having a more adequate gene expression, compared to the control group (G12). Therefore, the

coasting time of 60 h is preferable after subjecting ewes to the "Day 0 protocol" and ovarian stimulation. Implementing the appropriate coasting time to a superstimulation protocol can impact the current scenario of sheep IVEP.

Data availability

The data that support this study's findings are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Jasmine B.S. Pinheiro: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Lucas M. Figueira:** Conceptualization, Methodology, Data curation, Formal analysis, Writing – review & editing. **Lucas F.L. Correia:** Investigation, Data curation, Formal analysis, Writing – review & editing. **Thais A. Oliveira:** Investigation, Data curation, Writing – review & editing. **Viviane L. Brair:** Investigation, Data curation, Writing – review & editing. **Felipe F.P.C. Barros:** Methodology, Investigation, Data curation, Writing – review & editing. **Fabio O. Ascoli:** Methodology, Investigation, Data curation, Writing – review & editing. **Ribrio I.T.P. Batista:** Investigation, Data curation, Formal analysis, Writing – review & editing. **Felipe Z. Brandão:** Methodology, Project administration, Resources, Writing – review & editing. **Joanna M.G. Souza-Fabjan:** Project administration, Supervision, Conceptualization, Methodology, Investigation, Data curation, Formal analysis, 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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