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Dose and administration protocol for FSH used for ovarian stimulation affect gene expression in sheep cumulus–oocyte complexes

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Abstract. The present study evaluated the effect of four ovarian stimulation protocols on the follicular population and molecular status of cumulus–oocyte complexes (COCs). Twelve Santa Inês ewes (in a cross-over design) received 80 or 120 mg FSH alone in a multiple-dose (MD_{80} and MD_{120}) regimen or in combination with 300 IU equine chorionic gonadotrophin (eCG) in a one-shot (OS_{80} and OS_{120}) protocol. The follicular population, COC recovery rate, mean COCs per ewe and the rate of brilliant Cresyl blue-positive (BCB+) COCs were similar among treatments ($P > 0.05$). The expression of markers of oocyte competence (*ZAR1*, zygote arrest 1; *MATER*, maternal antigen that embryo requires; *GDF9*, growth differentiation factor 9; *BMP15*, bone morphogenetic protein 15; Bcl-2, B-cell lymphoma 2; BAX, Bcl-2 associated X protein) and the steroidogenic pathway (*ERα*, oestrogen receptor α; *LHr*, LH receptor; *FSHr*, FSH receptor; *STAR*, steroidogenic acute regulatory protein) was affected by stimulation. Specifically, the expression of markers of the steroidogenic pathway was reduced with increasing FSH dose in the OS protocol. FSH at a dose of 80 mg reduced the expression of *FSHr* and *ERα* in the OS versus MD protocol. Conversely, in MD protocol, only *LHr* was affected by increasing FSH dose. In conclusion, 80 mg FSH in the MD or OS protocol was sufficient to promote the development of multiple follicles and obtain fully grown (BCB+) oocytes. The MD protocol may be more appropriate for the production of better-quality oocytes.

Additional keywords: brilliant Cresyl blue, ewe, molecular biology, oocyte, superstimulation.

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Introduction

Assisted reproductive technologies (ARTs) have enabled the rapid propagation of embryos with genetically desirable characteristics for the animal breeding industry and the supply of biological material for basic research (Orozco-Lucero and Sirard 2014). Most ARTs, such as *in vitro* embryo production, transgenesis or cloning, require the recovery of a great number of good-quality oocytes (Souza-Fabjan *et al.* 2014*b*). In live females of monovular species, hormonal ovarian stimulation is performed as the first step in the application of these biotechnologies, because this stimulation increases the number of follicles available and thus the number of oocytes recovered (Gibbons *et al.* 2007; for a review, see Baldassarre 2012; Souza-Fabjan *et al.* 2013; Dias *et al.* 2013). However, oocyte quality is essential for the success of any ART technique (Dias *et al.* 2014).

Several ovarian stimulation protocols have been proposed for small ruminants. Different concentrations, origins (porcine or ovine) and degrees of purity of FSH, applied in either multiple doses (three to six) or as a 'one-shot' regimen, have been reported (Baldassarre *et al.* 1996, 2002; Berlinguer *et al.* 2004; Sousa *et al.* 2011). In general, although the short half-life of FSH (Laster 1972) makes it ineffective when administered alone in a single dose (Armstrong *et al.* 1994; Baldassarre *et al.* 1996), the use of either 80 or 120 mg FSH in multiple doses efficiently promotes follicle development (Sousa *et al.* 2011; for a review, see Baldassarre 2012; Santos *et al.* 2016). Indeed, the

use of 80 mg FSH in single dose with 300 IU equine chorionic gonadotropin (eCG), the so called 'one-shot' protocol, is efficient and often used in small ruminants due to the practical single handling (Baldassarre *et al.* 2002, 2003; Pierson *et al.* 2004; Teixeira *et al.* 2011; Baldassarre 2012). However, little is known about the molecular status of oocytes generated under different stimulatory protocols in small ruminants.

Exogenous gonadotrophin support leads physiological changes and affects intrafollicular regulatory mechanisms. Subordinate follicles can be rescued from atresia conditions, and multiple follicles can reach dominance and become able to ovulate, maintaining a high FSH plasma concentration (Adams *et al.* 1992; Yu *et al.* 2003; Pinto *et al.* 2018). Recent studies in cows using microarray analysis have shown that global gene expression in cumulus cells is altered by the use of stimulatory treatment (Dias *et al.* 2013) and can affect oocyte development competence (Blondin *et al.* 2002; Nivet *et al.* 2012; Lambrecque *et al.* 2013). The quality of the female gamete depends on the specific gene products (transcripts and proteins) stored in the cytoplasm during oocyte growth to support early development and during transcriptionally inactive periods, from maturation to activation of the embryonic genome (Krisher 2004; Sirard 2010).

The action of FSH in cumulus cells is mediated primarily by the cAMP-dependent protein kinase A (PKA) pathway (Hillier *et al.* 1994). Briefly, when FSH binds to its receptor (FSHr), adenylyl cyclase is activated, enabling cAMP production. The increased cAMP concentration activates PKA. Subsequently, the expression and/or activity of components of the steroidogenic machinery, such as steroidogenic acute regulatory protein (StAR), serve as the starting point for the cascade of cholesterol transformation (Wood and Strauss 2002; for a review, see Shimasaki *et al.* 2004). At the end of the cascade, 17β-oestradiol (E2) production enhances FSH action through a gradual increase in the expression of FSHr, LH receptor (LHr) and its own receptors, including ERα (for a review, see Couse and Korach 1999). In addition, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) produced by oocytes regulate FSH activity in the cumulus by modulating steroid production (Otsuka *et al.* 2005; Lahoz *et al.* 2013; Kona *et al.* 2016). Thus, the transcripts of genes, such as *GDF9*, *BMP15*, maternal antigen that embryo requires (*MATER*) and zygote arrest 1 (*ZAR1*), serve as potential markers of oocyte development competence (Bebbere *et al.* 2008; for a review, see Orozco-Lucero and Sirard 2014), as do intracellular proteins that regulate the apoptotic process by activating caspases, such as BAX (pro-apoptotic) and Bcl-2 (anti-apoptotic).

In ART, immature oocytes are often selected according to their morphological features (Almeida *et al.* 2011; Avelar *et al.* 2012; Souza-Fabjan *et al.* 2016). The brilliant Cresyl blue (BCB) test can also be used to determine oocyte growth (Turner *et al.* 2008; Catalá *et al.* 2011; Shabankareh *et al.* 2014). The germinal vesicle (GV)-stage oocyte is a relatively stable model in which to observe differences in accumulated mRNA levels with regard to its competence to form a viable embryo (Lambrecque *et al.* 2013). The present study tested three hypotheses: (1) that the stimulatory treatment, regardless of the protocol, affects the steroidogenic pathway and oocyte quality; (2) given an equivalent growth phase, the expression of gene transcripts related to steroidogenic machinery will increase in the cumulus–oocyte complex (COC) with increasing FSH dose in different regimens; and (3) molecular markers of oocyte quality may be favourably affected by the multiple-dose regimen because it may be more physiological. Thus, present study evaluated the effects of four stimulation protocols on the follicular population and molecular status of the COC, as evidenced by the gene expression of markers of oocyte quality (*GDF9*, *BMP15*, *MATER*, *ZAR1*, *Bcl-2* and *BAX*) and transcripts involved in steroidogenesis (*STAR*, *FSHr*, *LHr* and *ERα*) in Santa Inês ewes.

Materials and methods

Experimental design

Twelve Santa Ines ewes underwent ovarian stimulation using four different protocols (Sousa *et al.* 2011; Baldassarre 2012) in a cross-over design (four treatments, three ewes per treatment, four replicates). There was a 21-day interval between replicates. The one-shot protocol with 80 mg FSH plus 300 IU eCG was considered the standard treatment (Baldassarre *et al.* 2002, 2003; Baldassarre 2012). Ovarian dynamics were monitored by ultrasonography from sponge removal (oestrus synchronisation) up to laparoscopic ovum pick-up (LOPU). Four LOPU sessions were performed, and the COCs recovered were graded morphologically (see below) and tested using BCB. BCB-positive (BCB^+) COCs were used for gene expression analysis. The concentration of oestradiol (E2) in the follicular fluid was determined. In addition, two ewes that had not received any treatment underwent LOPU in the last two sessions ($n = 4$). COC recovery was graded and tested using BCB. The BCB^+ COCs from the untreated ewes were used as control calibrators in the gene expression analyses.

Location, conditions and animal care

The study was performed from September to December 2016 at the Unidade de Pesquisa Experimental em Caprinos e Ovinos (UNIPECO) da Universidade Federal Fluminense, in Cachoeiras de Macacu, Rio de Janeiro, Brazil (22°27′45″S). All procedures were approved by the local Ethics Committee for Animal Use of the Universidade Federal Fluminense (Protocol #721/2015) and conducted under the ethical principles of the Sociedade Brasileira de Experimentação Animal. Fourteen clinically healthy adult multiparous Santa Inês ewes (mean (± s.d.) bodyweight 53 ± 5 kg and body condition score 3.7 ± 0.3) were housed and received chopped elephant grass (*Pennisetum purpureum*) and 200 g concentrate (12% crude protein) per animal twice daily; and water and mineralised salt were provided *ad libitum*.

Hormonal stimulation

Oestrus synchronisation was performed with intravaginal sponges containing 60 mg medroxyprogesterone acetate (Progespon; Schering Plough Animal Health) used for 6 days. One day before sponge removal, 300 IU eCG (Novormon 5000; MSD Animal Health) and 0.12 mg cloprostenol sodium (Estron; Tecnopec) were administered intramuscularly (i.m.), whereas

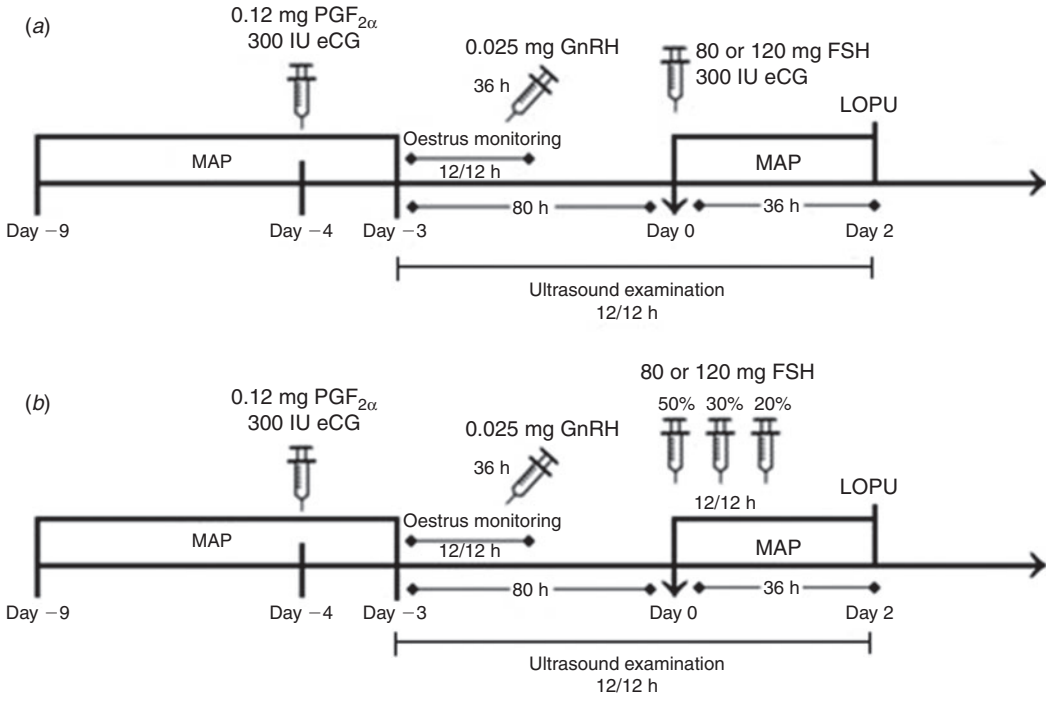

Fig. 1. Schematic diagram of the experimental design. Oestrus synchronisation treatment was applied in all groups to reach ovulation on Day 0. At Day 0, a new progesterone sponge was inserted and ovarian stimulation was performed with either (*a*) only one dose of FSH (either 80 or 120 mg) and 300 IU equine chorionic gonadotrophin (eCG), the so-called one-shot protocol (OS_{80} and OS_{120}) or (*b*) multiple decreasing doses of FSH (totalling 80 or 120 mg; MD_{80} or MD_{120} respectively). $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; MAP, medroxyprogesterone acetate; LOPU, laparoscopic ovum pick-up; GnRH, gonadotrophin-releasing hormone.

0.025 mg lecirelin (Gestran Plus; Tecnopec) was administered 36 h after progestagen withdrawal (Balaro *et al.* 2016). Every 12 h after sponge removal, ewes were subjected to oestrus detection with teaser rams for evaluation of sexual behaviour. Stimulation was initiated (considered Day 0) 80 h after sponge removal and 36 h before LOPU. Moreover, a new sponge was placed in the stimulation (first FSH application), which was at 80 h after first sponge removal. The following stimulation protocols were used: multiple doses (MD) of 80 or 120 mg FSH (Folltropin-V; Bioniche Animal Health) administered in three decreasing doses (50%, 30% and 20%) every 12 h (MD_{80} and MD_{120} respectively) and one-shot (OS) dosing consisting of 80 mg FSH (standard protocol) or 120 mg FSH plus 300 IU eCG (OS_{80} and OS_{120} respectively). Fig. 1 illustrates the experimental treatments and procedures used in the present study.

Ovarian assessment

Transrectal ultrasonography was performed every 12 h from the day of first sponge removal to the day of LOPU. Ovulatory parameters (ovulation and the size of the largest follicle) and the number, position and diameter of the ovarian follicles were recorded. Follicles were classified according to their size as small (diameter <3.0 mm), medium (diameter 3–5.0 mm) or large (diameter >5.0 mm). The follicular population was observed immediately before the start of ovarian stimulation (Day 0) and immediately before LOPU (Day 2). Evaluations were performed using an ultrasound (SonoScape) equipped with a 7.5-MHz linear transducer in B-mode. Colour Doppler mode was used to assess the status of the corpora lutea (CL; regression or formation after ovulation at Day 0).

COC recovery

Donor ewes were deprived of food for 36 h and water for 24 h before laparoscopic procedures. Pre-anaesthetic medication consisted of the administration of 0.2 mg kg^{-1}, i.v., diazepam (Teuto), 0.1 mg kg^{-1}, i.v., acepromazine (Acepran; Vetnil) and 0.4 mg kg^{-1}, i.m., morphine (Dolo Moff). Anaesthesia was induced with 6 mg kg^{-1} ketamine (Dopalen; Vetbrands) and 10 mg of 1% propofol (Profolen; Balusiegel) i.v. and maintained with isoflurane (Forane; Abbott Laboratórios) via endotracheal intubation. For local anaesthesia, 2% lignocaine (Lidovet; Bravet) was injected. LOPU was performed according to Baldassarre *et al.* (2003). Briefly, visible follicles (2–8 mm) were aspirated using a 20-G needle with a short bevel connected to a 50-cm cannula, which was connected to a silicone cork (Handle Cook) and a Falcon tube (50 mL). The aspiration pressure was regulated at 36 mmHg by a vacuum pump (WTA). The aspiration medium was TCM-199 (M2520; Sigma-Aldrich) supplemented with 20 IU mL^{-1} heparin (Calbiochem 375095), 0.2 mM pyruvate (P4562; Sigma), 100 IU penicillin, 100 μg mL^{-1} streptomycin, 0.25 μg mL^{-1} amphotericin (A5955; Sigma) and 10% bovine serum albumin (BSA; A9647; Sigma). Initially, 4 mL

aspiration medium was added to the collection tube, with another 4 mL added later to wash the aspiration system, giving a final volume of 8 mL.

COC grading and BCB test

COCs were isolated under a stereomicroscope and graded based on visual assessment of morphology (cellular layers and cytoplasmic uniformity) according to Souza-Fabjan *et al.* (2016) as follows: Grade I/II, good; Grade III, acceptable; Grade IV, poor. For inference of developmental competence, viable COCs (Grades I–III) from each treatment were washed once in Dulbecco's modified phosphate buffered saline (DMPBS, Biodux®) supplemented with 10% BSA (A9647, Sigma). In sequence, COCs were exposed to 26 μM BCB (B5388, Sigma) diluted in DMPBS with 10% BSA and 0.2 mM pyruvate (P4562; Sigma) for 60 min on a hot plate at 37°C and ambient atmosphere. Then, COCs were washed twice in DMBPS with 10% BSA, placed in polyvinyl alcohol (PVA; P8136; Sigma) for 5 min at 37°C and classified according to oocyte cytoplasm staining as BCB^+ (blue cytoplasm) or BCB^- (colourless cytoplasm). Pools of 10 BCB^+ COCs from the same treatment were recovered (with a minimal amount of medium) at once with a pipette calibrated at 2 μL, transferred to DNAse- and RNAse-free cryotubes, snap frozen and stored in a liquid nitrogen canister for later gene expression analysis.

Follicular fluid E2 concentrations

Following the LOPU procedure, the collection tube containing 8 mL follicular contents was left to rest for decantation. After 5 min, 300 μL supernatant was aliquoted in triplicate and stored at −20°C. The samples from each ewe were aliquoted separately. Radioimmunoassay (RIA) was used to measure E2 concentrations in the follicular fluid using a commercially available kit (17β-Oestradiol – ImmuChem Double Antibody; MP Biomedicals) according to the manufacturer's instructions. The samples were analysed in a single assay, with a standard dilution of 1 : 2 and an intra-assay CV of 7%. After determination of E2 concentrations, they were corrected mathematically by multiplying the observed concentration by the dilution factor.

Gene expression analysis

To assess the abundance of mRNA encoding protein markers of oocyte competence (i.e. MATER, ZAR1, BMP15, GDF9, BAX, Bcl-2) and the steroidogenic pathway (i.e. FSHr, LHr, STAR and ERα), a control (unstimulated ewe) was used as a calibrator to compare parameters of physiological molecular status with each treatment.

Samples were analysed by real-time quantitative polymerase chain reaction (qPCR) after reverse transcription according to Batista *et al.* (2014). Total RNA was extracted from 3 pools of 10 BCB^+ COCs per group using an RNeasy Micro kit (Qiagen) according to the manufacturer's instructions and reverse transcribed using a Superscript III kit (Invitrogen). The total RNA from each pool for treatment group was quantified using 1 μL sample and an ND-100 spectrophotometer (NanoDrop Products). Relative quantification by real-time qPCR was performed in duplicate using a commercially available SYBR green kit (Power SYBR Green; Applied Biosystems), 400 ng cDNA, nuclease-free water and specific primers (Table 1) for every reaction. Template cDNA was denatured at 95°C for 15 min, followed by 45 cycles of denaturation at 94°C for 15 s, gene-specific primer annealing temperature for 30 s and elongation at 60°C for 30 s. After each PCR run, melting curve analysis was performed to confirm that a single specific product was generated. No-template controls (NTCs), comprising the PCR reaction mix without DNA template, were also run with each primer to confirm the absence of contamination.

Statistical analysis

Outcome variables related to sexual behaviour, ovulatory parameters, follicular population, recovery rate, total number of COCs, morphological quality, BCB staining of the COCs per ewe and E2 concentration were evaluated for normality using the Lilliefors test. The main effects of FSH dose and administration regimen, as well as the interaction between these parameters, were assessed by analysis of variance (ANOVA), and the significance of differences between mean values was determined using Tukey's test. Data that were not normally distributed (i.e. follicular population) were subjected to square root transformation before ANOVA. For gene expression analysis, primer efficiency was tested using LinRegPCR software (Qiagen; Ramakers *et al.* 2003) for each reaction. Relative quantification was performed using REST software (Qiagen; Pfaffl *et al.* 2002) based on comparative Ct quantification. The values are given as differences relative to the calibrator. Statistical analyses were performed using a statistical analysis system program (SAEG 9.0; Federal University of Viçosa). Differences were considered significant at two-tailed $P < 0.05$. Data are given as the mean ± s.d.

Results

Oestrus synchronisation and the follicular wave

After oestrus synchronisation with the first progestogen sponge (from Day −3 to Day 0 in Fig. 1), sexual behaviour and ovulatory parameters were evaluated. Overall, the oestrous response rate was 85% (41/48), the interval from sponge removal to oestrus was 27.5 ± 10.8 h, the interval from oestrus to ovulation was 26.4 ± 11.4 h, the duration of oestrus was 32.0 ± 13.9 h, the ovulation rate was 98% (47/48), the number of ovulations was 1.2 ± 0.4 and the largest follicle diameter was 6.4 ± 0.9 mm. These parameters did not differ among ewes from the four experimental groups.

Ovarian response to the stimulation treatments

Data regarding the different follicular populations are given in Table 2. At Day 0, there were no differences were among treatments groups with regard to the number of follicles in each category (small, medium and large). In the OS_{80}, OS_{120} and MD_{120} groups, the number of small follicles was greater than the number of medium follicles, which was greater than the number of large follicles. In the MD_{80} group, the number of small follicles was also greater than the number of medium and large follicles, which did not differ from each other. On Day 2, there were no effects of FSH dose and administration regimen, or their

Table 1. Sequences of the specific primers used in the analysis of gene expression in brilliant Cresyl blue-positive cumulus–oocyte complexes (BCB+) recovered from immature sheep by laparoscopy after different ovarian stimulation protocols

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *ZAR1*, zygote arrest 1; *MATER*, maternal antigen that embryo requires; *GDF9*, growth differentiation factor 9; *BMP15*, bone morphogenetic protein 15; *ERα*, oestrogen receptor α; *LHr*, LH receptor; *FSHr*, FSH receptor; *STAR*, steroidogenic acute regulatory protein

Gene	Primer sequence (5′–3′)	Annealing temperature (°C)	Size (bp)	Reference
GAPDH	Forward: GGG AAA TCG TGC GTG ACA TTA AG Reverse: TGT GTT GGC GTA AGG TCT TTG	60	273	Hogg *et al.* (2011)
ZAR1	Forward: CAC TGC AAG GAC TGC AAT ATC Reverse: CAG GTG ATA TCC TCC ACT C	60	137	Bebbere *et al.* (2008)
MATER	Forward: CAG CCT CCA GGA GTT CTT TG Reverse: GAC AGC CTA GGA GGG TTT CC	59	212	Bebbere *et al.* (2008)
GDF9	Forward: CAG ACG CCA CCT CTA CAA CA Reverse: CAG GAA AGG GAA AAG AAA TGG	58	198	Bebbere *et al.* (2008)
BMP15	Forward: GGG TTC TAC GAC TCC GCT TC Reverse: GGT TAC TTT CAG GCC CAT CAT	59	273	Bebbere *et al.* (2008)
BAX	Forward: CCT GGG ATC TTG AAA CTC TCC TT Reverse: CTG AGC CAG GCT GAA ATC AAA A	60	566	Chakravarthi *et al.* (2015)
Bcl-2	Forward: GCC GAG TGA GCA GGA AGA C Reverse: GTT AGC CAG TGC TTG CTG AGA	60	214	Chakravarthi *et al.* (2015)
ERα	Forward: GAA TCT GCC AAG GAG ACT CG Reverse: CCT GAC AGC TCT TCC TCC TG	60	187	Hogg *et al.* (2011)
LHr	Forward: TCC GAA AGC TTC CAG ATG TT Reverse: GAA ATC AGC GTT GTC CCA TT	60	199	Hogg *et al.* (2011)
FSHr	Forward: TAA GCA CTT GCC AGC TGT TC Reverse: CTC ATC GAG TTG GGT TCC AT	60	196	Hogg *et al.* (2011)
STAR	Forward: GCA TCC TCA AAG ACC AGG AG Reverse: CTT GAC ACT GGG GTT CCA CT	60	194	Hogg *et al.* (2011)

Table 2. Ovarian follicular population immediately before (Day 0) and after (Day 2) hormonal stimulation treatments in Santa Inês ewes (mean ± s.d.)

Data are the mean ± s.d. ($n = 12$ ewes per treatment). Within rows, A,B values with different uppercase superscript letters differ significantly ($P < 0.05$) within the same day. OS_{80}, 80 mg FSH plus 300 equine chorionic gonadotrophin (eCG) applied in one-shot; OS_{120}, 120 mg FSH plus 300 IU eCG applied in one-shot; MD_{80}, 80 mg FSH applied in multiple decreasing doses; MD_{120}, 120 mg of FSH applied in multiple decreasing doses

Treatment	Day 0			Day 2		
	Small (<3 mm)	Medium (3–5 mm)	Large (>5 mm)	Small (<3 mm)	Medium (3–5 mm)	Large (>5 mm)
OS_{80}	8.5 ± 3.6^{A}	4.3 ± 2.0^{B}	0.1 ± 0.3^{C}	3.5 ± 3.5^{A}	5.7 ± 2.4^{B}	1.3 ± 1.2^{A}
OS_{120}	9.2 ± 3.2^{A}	2.8 ± 1.2^{B}	0.1 ± 0.3^{C}	3.4 ± 2.4^{A}	7.9 ± 2.3^{B}	0.9 ± 1.4^{C}
MD_{80}	9.4 ± 3.3^{A}	2.4 ± 2.4^{B}	0.3 ± 0.5^{B}	3.8 ± 3.4^{A}	8.1 ± 2.2^{B}	0.5 ± 0.9^{C}
MD_{120}	8.3 ± 5.1^{A}	3.3 ± 1.8^{B}	0.1 ± 0.3^{C}	3.3 ± 2.0^{A}	7.8 ± 3.6^{B}	0.8 ± 1.0^{C}

interaction, on the number of follicles in any category. In the OS_{120}, MD_{80} and MD_{120} groups, the number of medium follicles was greater than the number of small follicles, which, in turn, was larger than the number of large follicles. Even in the OS_{80} group, the number of medium follicles was greater than the number of of small and large follicles, which did not differ from each other.

COC recovery and BCB test

Quantitative and qualitative aspects of COCs produced from the four hormone treatment protocols are given in Table 3. There were no differences among treatments in the parameters evaluated. However, there was an effect of the administration regimen on COC morphology, whereby MD resulted in a greater number of Grade II COCs than the OS regimen. There was no significant difference in the amount of the morphologically good-quality COCs (Grades I + II) among the OS_{80} (56%; 39/70), OS_{120} (66%; 43/65), MD_{80} (74%; 61/82) and MD_{120} (70%; 64/91) treatment groups. Regardless of the regimen used (i.e. OS or MD), when taking the dose of FSH only into consideration (i.e. 80 and 120 mg FSH), the total number of COCs per ewe was 6.3 ± 0.9 and 6.5 ± 1.8 respectively, the number of viable COCs

Table 3. Effects of different ovarian stimulation treatments on the quantity and quality (morphology and brilliant Cresyl blue test) of immature cumulus–oocyte complexes (COCs) recovered by laparoscopy in Santa Inês ewes (mean ± s.d.)

Unless indicated otherwise, data are the mean ± s.d. ($n = 12$ ewes per treatment). Within columns, values with different superscript letters differ significantly ($P < 0.05$). COCs were graded based on visual assessment of morphology (cellular layers and cytoplasmic uniformity) according to Souza-Fabjan *et al.* (2016) as follows: Viable COCs were considered to be those in Grades I–III, whereas good-quality COCs were considered those in Grades I and II. OS_{80}, 80 mg FSH plus 300 IU equine chorionic gonadotrophin (eCG) applied in one-shot; OS_{120}, 120 mg FSH plus 300 IU eCG applied in one-shot; MD_{80}, 80 mg FSH applied in multiple decreasing doses; MD_{120}, 120 mg of FSH applied in multiple decreasing doses; BCB+, brilliant Cresyl blue-positive COCs

Treatment	% Recovery	Total COCs	Viable COCs	Good-quality COCs	BCB+ (%)
OS_{80}	65 (70/107)	5.8 ± 0.6	5.4 ± 0.9	3.3 ± 0.7[b]	72
OS_{120}	59 (65/110)	5.4 ± 1.0	5.3 ± 0.8	3.6 ± 0.6[b]	88
MD_{80}	68 (82/120)	6.8 ± 0.9	6.6 ± 0.9	5.1 ± 0.4[a]	80
MD_{120}	70 (91/130)	7.6 ± 1.9	7.3 ± 1.3	5.3 ± 2.3[a]	76

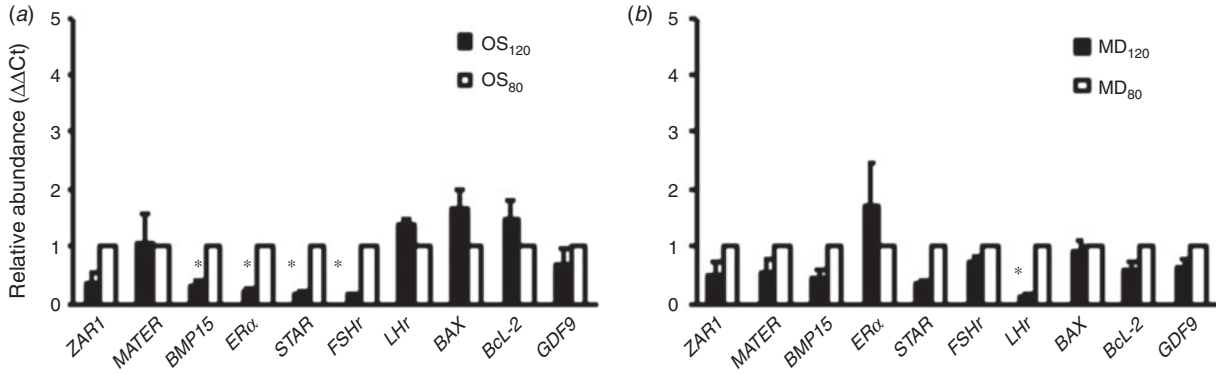

Fig. 2. Effect of FSH dose on the gene expression profile in brilliant Cresyl blue-positive cumulus–oocytes complexes (COC). Relative gene expression is shown in COCs derived from different hormone protocols for ovarian stimulation (OS_{80}, 80 mg FSH plus 300 equine chorionic gonadotrophin (eCG) applied in one-shot; OS_{120}, 120 mg FSH plus 300 IU eCG applied in one-shot; MD_{80}, 80 mg FSH applied in multiple decreasing doses; MD_{120}, 120 mg of FSH applied in multiple decreasing doses). Data show mean ± s.d. fold changes relative to the calibrator (= 1), which was the OS_{80} data in (*a*) and MD_{80} data in (*b*). *$P < 0.05$ compared with reference gene GAPDH, glyceraldehyde 3-phosphate dehydrogenase *ZAR1*, zygote arrest 1; *MATER*, maternal antigen that embryo requires; *GDF9*, growth differentiation factor 9; *BMP15*, bone morphogenetic protein 15; *ERα*, oestrogen receptor α; *LHr*, LH receptor; *FSHr*, FSH receptor; *STAR*, steroidogenic acute regulatory protein.

per ewe was 6.0 ± 1.0 and 6.3 ± 1.6 respectively and the number of BCB^+ COCs per ewe was 4.6 ± 1.5 and 5.1 ± 1.1.

Gene expression

Regardless of the dose and administration regimen, the ovarian stimulation protocols altered the gene expression pattern of markers of the steroidogenic pathway and oocyte quality in COCs with complete exponential growth (i.e. BCB^+ COCs) compared with control. The steroidogenic pathway genes were downregulated, except for *FSHr* in the OS_{80} group, which was not differentially expressed. Compared with control, markers of oocyte quality markers were upregulated in the OS_{80} (*ZAR1*, *BMP15*, *GDF9*, *BAX* and *Bcl-2*), OS_{120} (*GDF9*, *BAX* and *Bcl-2*), MD_{80} (*MATER*, *ZAR1*, *BMP15*, *GDF9*, *BAX* and *Bcl-2*) and MD_{120} (*ZAR1*, *GDF9*, *BAX* and *Bcl-2*) groups.

Different doses of FSH (80 vs 120 mg) were compared within the same administration protocol, and the results are shown in Fig. 2. Steroidogenic pathway transcripts (*FSHr*, *ERα* and *STAR*) and *BMP15* were downregulated in the OS_{120} group (Fig. 2*a*). However, only *LHr* was downregulated in the MD_{120} group (Fig. 2*b*).

Different administration regimens (OS vs MD) using the same FSH dose were compared, and the results are shown in Fig. 3. The steroidogenic pathway (*FSHr* and *ERα*) was downregulated in the OS_{80} group, whereas *LHr* was downregulated in the MD_{120} group.

Follicular fluid E2 concentrations

The E2 concentrations in the aspirated fluid did not differ significantly ($P > 0.05$) among all four treatment groups. The mean, minimum and maximum E2 concentrations were as follows: 11.511 ± 11.991, 1.561 and 43.680 ng dL^{-1} respectively in 98 follicles in the OS_{80} group; 5.235 ± 2.406, 817 and 9162 ng dL^{-1} respectively in 110 follicles in the OS_{120} group; 5.217 ± 4.783, 1.439 and 18.862 ng dL^{-1} respectively in 120 follicles in the MD_{80} group; and 7.544 ± 6.893, 585 and 25.405 ng dL^{-1} respectively in 108 follicles in the MD_{120} group. All data were within the maximum and minimum points of the standard curve.

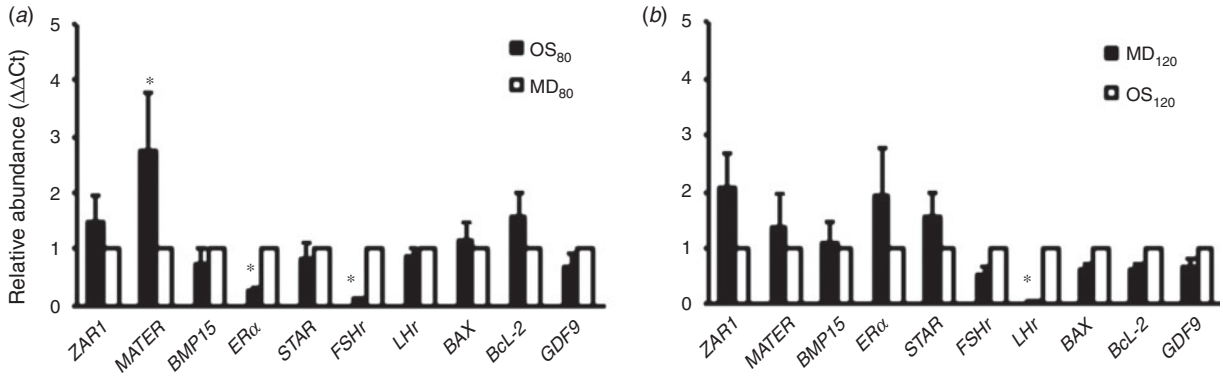

Fig. 3. Effect of FSH administration strategy on the gene expression profile in brilliant Cresyl blue-positive cumulus–oocyte complexes (COC). Relative gene expression is shown in COCs derived from different hormone protocols for ovarian stimulation (OS_{80}, 80 mg FSH plus 300 equine chorionic gonadotrophin (eCG) applied in one-shot; OS_{120}, 120 mg FSH plus 300 IU eCG applied in one-shot; MD_{80}, 80 mg FSH applied in multiple decreasing doses; MD_{120}, 120 mg of FSH applied in multiple decreasing doses). Data show mean ± s.d. fold changes relative to the calibrator (= 1), which was the MD_{80} data in (*a*) and OS_{120} data in (*b*). *$P < 0.05$ compared with reference gene GAPDH, glyceraldehyde 3-phosphate dehydrogenase *ZAR1*, zygote arrest 1; *MATER*, maternal antigen that embryo requires; *GDF9*, growth differentiation factor 9; *BMP15*, bone morphogenetic protein 15; *ERα*, oestrogen receptor α; *LHr*, LH receptor; *FSHr*, FSH receptor; *STAR*, steroidogenic acute regulatory protein.

Discussion

The effects of four stimulation protocols on the follicular population and molecular status of COCs were evaluated in Santa Inês ewes. As expected after synchronisation, sexual behaviour and ovulatory parameters were not different among the groups, because the same synchronisation protocol has been used until recently. High oestrus and ovulation response rates (85% and 98% respectively) were observed in the present study, as also reported by Balaro *et al.* (2016), who obtained 78% and 89% oestrus and ovulation rates respectively using the same synchronisation protocol. These results demonstrate that the follicular population at Day 0 (immediately before the start of stimulation) was similar in all groups, and the number of small follicles was significantly greater than the number of medium and large follicles. At Day 2 (the day of LOPU), neither the FSH dose nor the administration regimen had any effect on the follicular population, as reported by Baldassarre *et al.* (2002) but in contrast with the findings of Sousa *et al.* (2011). Regardless of the stimulation strategy, FSH induced a significant increase in the number of medium follicles and a decrease in the number of small follicles (Table 2), consistent with the findings of Yu *et al.* (2003), Berlinguer *et al.* (2004) and Gibbons *et al.* (2007). In small ruminants, there is a positive correlation between medium-sized (3–5 mm) COCs and better maturation, cleavage and blastocyst rates, and this diameter is the preferential size for aspiration for *in vitro* production (IVP; Lahoz *et al.* 2013; Souza-Fabjan *et al.* 2014*a*). A study in cows (Nivet *et al.* 2012) demonstrated that COCs from medium-sized follicles (7–10 mm) had greater developmental competence than COCs from small (5–6 mm) and large (>10 mm) follicles. In addition, there was a positive correlation between follicle size (up to 8 mm) and developmental competence, but, for reasons unknown, the correlation was negative in large follicles (Nivet *et al.* 2012).

An effect of the administration regimen on COC morphological quality was observed in the present study, whereby MD treatments produced a greater amount of Grade II COCs than did OS treatments. Curiously, a similar effect was reported by Santos *et al.* (2016). The rate of BCB^+ COCs was not different among the groups. The relationship between viable COCs (Grades I–III) and the BCB^+ rate demonstrated that the competence of Grade III COCs (denuded but with a homogeneous cytoplasm) may be satisfactory, as proposed by Souza-Fabjan *et al.* (2016). The BCB test in IVP programs has been used to select oocytes that have completed the exponential growth phase and thus have theoretically acquired the capability to develop *in vitro*, promoting the selection of a more homogeneous oocyte pool (Catalá *et al.* 2011; for a review, see Paramio and Izquierdo 2016). In a previous study, the percentage of immature BCB^+ COCs from small follicles was lower than that from medium or large follicles (Shabankareh *et al.* 2014). In another study, BCB^+ COCs from small and large follicles exhibited similar developmental competence, despite differences in their meiotic and cytoplasmic maturation (Yang *et al.* 2016).

The results of the present study provide new information on the dose effect and strategy of FSH administration on the relative expression patterns of genes associated with steroidogenesis (*ERα*, *STAR*, *FSHr* and *LHr*) and markers of quality (*ZAR1*, *MATER*, *BMP15*, *GDF9*, *BAX* and *Bcl-2*) in COCs. Regardless of the dose of FSH and strategy of administration, the genes involved in steroidogenesis were downregulated, whereas those that were markers of quality were upregulated compared with the unstimulated control calibrator. These findings suggest that the increase in circulating FSH concentration induces a reduction in the expression of FSHr and consequently other receptors and enzymes of the PKA pathway as a mechanism to maintain healthy intrafollicular oestrogen levels. Consistent with this hypothesis, the intrafollicular oestrogen concentration was similar among the different treatment groups. According to Dias *et al.* (2013), multiple preovulatory follicles that develop after stimulation are not typical dominant follicles.

In all, 470 genes are differentially expressed in granulosa cells (GCs) from stimulated versus unstimulated follicles (Dias *et al.* 2013). Stimulation primarily affects cellular growth and development functions, delaying GC differentiation due the upregulation of genes associated with cell growth and proliferation, which causes a blunted response to LH signalling in GCs from stimulated follicles compared with GCs from unstimulated dominant follicles, where these genes are downregulated. Nevertheless, not all functions are equally affected (e.g. steroidogenic function) because the intrafollicular E2 concentration and E2 : progesterone (P4) ratio were similar between stimulated and unstimulated follicles (Dias *et al.* 2013).

The improvement in the gene expression of quality markers, such as *GDF9* (in all treatment groups) and *BMP15* in the OS_{80} and MD_{80} groups, justifies the reduction in the steroidogenic pathway. GDF9 and BMP15 synergistically regulate FSH activity in the proliferation of and steroid production by GCs, playing crucial roles in normal follicular development and following a stage-specific pattern of expression *in vivo* (Otsuka *et al.* 2005; Bebbere *et al.* 2008; Lahoz *et al.* 2013; Kona *et al.* 2016). Members of the transforming growth factor β (TGFB) superfamily exert a luteinisation inhibitor action in GCs (for a review, see Shimasaki *et al.* 2004). *In vitro*, GDF9 alone stimulates basal steroidogenesis in preovulatory GCs, but in coculture with FSH, GDF9 induces the dose-dependent suppression of cAMP production, and consequent P4 and E2 synthesis attenuate FSH-dependent LHr expression (Vitt *et al.* 2000). BMP15 inhibits FSHr expression and cAMP signalling, thereby reducing the expression of PKA pathway components, including StAR protein and LHr (Otsuka *et al.* 2001). The expression of GDF9 and BMP15 is FSH independent (for a review, see Shimasaki *et al.* 2004), but in the present study GDF9 was affected by exogenous FSH, and BMP15 was affected by FSH dose (80 mg). FSH may play a role in the modulation of the cumulus response to oocyte-secreted factors such as GDF9 and BMP15 (Khan *et al.* 2015).

Other markers of oocyte quality that were upregulated in stimulated BCB^+ COCS included *MATER* (MD_{80}) and *ZAR1* (MD_{80}, OS_{80} and MD_{120}). MATER and ZAR1 are required for early development before zygotic genome activation, and these proteins have similar expression patterns. The abundance of mRNA transcripts of *MATER* and *ZAR1* is a good predictor of developmental competence in immature GV oocytes (Bebbere *et al.* 2008; Urrego *et al.* 2015), indicating that stimulation protocols can enhance COC quality depending on the dose and administration strategy (e.g. 80 mg FSH showed better modulation of both maternal effect genes in the MD_{80} protocol and of *ZAR1* in the OS_{80} protocol). However, 120 mg FSH administered in the MD regimen also increased *ZAR1* expression. This confirmed our hypothesis that MD is more suitable for producing better-quality COCs. In addition, we considered apoptosis proteins, namely BAX (pro-apoptotic) and Bcl-2 (anti-apoptotic), as markers of COC quality. In all stimulation protocols evaluated in the present study, BAX and Bcl-2 were abundantly expressed. However, the relative balance between the different pro- and anti-apoptotic proteins, reflecting the formation of homodimers and heterodimers (neutralisation), defines the route of programmed cell death (for a review, see Zörnig *et al.* 2001). The findings of the present study suggested the neutralisation of the molecular mechanisms of apoptosis. Apoptosis is the mechanism responsible for promoting atresia. A study using microarray analysis of stimulated follicles demonstrated that despite stimulation rescue of follicles from atresia, some molecules involved with that pathway continue to be triggered; in contrast, other known anti-atresia markers were upregulated after stimulation (Dias *et al.* 2013).

Comparing different doses of FSH (80 vs 120 mg) within the same administration regimen (Fig. 2) revealed that the steroidogenic pathway (*FSHr*, *ERα* and *STAR*) and *BMP15* (quality marker) were reduced by the increased FSH dose in the OS protocol. Comparing the different regimens (OS vs MD) using the same FSH dose (Fig. 3) revealed that the steroidogenic pathway (*FSH* and *ERα*) was reduced in the OS_{80} group. Conversely, in MD, only *LHr* was reduced in both comparisons. These findings indicated that a lower FSH concentration (80 mg) causes a better response to steroidogenic pathway signalling, and the MD regimen promoted less suppression of the expression of these genes. The eCG synergism did not contribute to the effect on FSHr expression in the OS protocol, as demonstrated by the comparison between different regimens at the same FSH dose. eCG has an activity similar to pituitary gonadotropins and even has high affinity for FSHr and LHr (Wei *et al.* 2016). The long half-life of eCG enabled the continued development of the follicles whose growth had been initiated by the bolus injection of FSH (Armstrong *et al.* 1994; Baldassarre *et al.* 1996), as used in the OS regimen. FSHr is essential for FSH action and is primarily expressed by cumulus and mural granulosa cells in sheep (Tisdall *et al.* 1995). FSHr expression in the present study was reduced with increasing FSH dose, consistent with the findings of Santos *et al.* (2016). The reduced expression of LHr in the MD_{120} compared with OS_{120} group may indicate a better quality of COCs with MD. The abundance of LHr expression in GCs from immature COCs was associated with the poor quality of the oocytes (Vigone *et al.* 2015; Santos *et al.* 2016), because LHr expression has been reported as a parameter to differentiate immature oocytes as competent or not for *in vitro* development (Vigone *et al.* 2015). The suppressed expression of *STAR* in OS_{120} compared with OS_{80} likely indicates a mechanism in which GDF9 and/or BMP15 inhibit early luteinisation. Members of the TGFB family act through this mechanism to maintain the E2 : P4 ratio, because a premature increase in P4 levels is correlated with follicular atresia (Zheng *et al.* 2008). *ERα* was reduced in the OS_{120} compared with OS_{80} group and in the OS_{80} compared with MD_{80} group. This reduction may be a consequence of the reduced FSHr and PKA pathways. Increased expression of E2 receptors has been associated with the attenuation of apoptosis and follicular atresia (for a review, see Couse and Korach 1999). Perhaps the MD_{80} protocol produced better-quality COCs than the other treatments tested, despite the similar E2 concentrations in all treatments.

In conclusion, 80 mg FSH in the MD or OS protocols was sufficient to promote the development of multiple follicles available for COC recovery by LOPU, generating COCs with good morphological quality and fully grown (BCB^+) that are predicted to be more competent for *in vitro* development. The MD regimen may be more appropriate for producing better-quality oocytes.

Conflicts of interest

The authors declare no conflicts of interest.

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