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Exogenous progestogens differentially alter gene expression of immature cumulus–oocyte complexes in sheep

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ABSTRACT

This study evaluated the role of progesterone (P_4) and medroxyprogesterone acetate (MAP) on the molecular status of immature cumulus–oocyte complexes (COCs) and the implications for oocyte quality in sheep. The number of viable COCs per ewe and the rate of COCs screened for developmental competence by brilliant cresyl blue positive (BCB^+) were similar ($P > 0.05$), respectively, across treatments (P_4: 7.7 ± 0.7 and 4.7 ± 1.2; MAP: 5.7 ± 1.0 and 3.5 ± 2.3; and control: 5.7 ± 1.1 and 3.6 ± 2.4). The COCs' gene expression was altered by exogenous progestogens compared with the control group: markers of steroidogenic pathway (FSH receptor [*FSHr*], LH receptor [*LHr*], and estradiol receptor α) and of quality (zygote arrest 1, growth differentiation factor 9, and B-cell lymphoma 2) were in abundance in P_4 ($P < 0.05$). In addition, reelin protein (*RELN*) was downregulated, and *Bcl-2* was upregulated in MAP ($P < 0.05$). In the P_4 vs MAP comparison, *FSHr, LHr*, and *RELN* genes were upregulated ($P < 0.05$) in the P_4 group. In conclusion, P_4 and MAP promoted dissimilar effects on transcriptome profiling of immature BCB-selected COCs, possibly due to the differences in the chemical structure of progestogens and concentrations of serum P_4. Exogenous P_4 impacted positively on the profile of genes related to oocyte quality.

1. Introduction

Hormonal manipulation of ovarian function using the association of gonadotropins and progestogens is widely applied in assisted reproductive technologies to improve oocyte recovery in live females of monovular species [1–3]. Stimulatory protocols with FSH increase follicular population [4,5] and alter global gene expression in oocyte and cumulus cells [6–8]. Progestogen support during gonadotropin treatments is used to inhibit LH pulse frequency, allowing regression of dominant follicle(s) by atresia, and thus avoiding ovulation [2,9].

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Progestogens (P_4) are natural or synthetic steroids that exert P_4-like activity. Progesterone is the only natural progestogen and is produced primarily by corpus luteum after ovulation. Progesterone analogs, such as medroxyprogesterone acetate (MAP), simulate the therapeutic effects of P_4, despite some differences in chemical structure. These differences result in different actions at the cellular level [10,11]. Progesterone has an important role in mammalian oocyte maturation, but its impact on oocyte quality has not yet been properly clarified [12]. In addition, no information is available about the effect of P_4 vs MAP during antral follicular growth on the gene expression of immature cumulus–oocyte complexes (COCs).

Recent evidence using intravaginal P_4 administration during FSH stimulation of the first wave of the estrous cycle showed that sheep embryo yield is improved in *in vivo* [13] and in vitro [14] production systems. The authors demonstrated that this improvement was associated with a positive effect of P_4 on oocyte developmental competence [14]. In contrast, Bartlewski et al [15] evaluated the use of P_4 vs MAP during a conventional superovulation protocol and observed no difference in terms of number of ovulations and embryos recovered. Conversely, some studies reported that exogenous progestogens had a deleterious effect on in vitro embryo production [1], mainly in long-term based treatments [3]. Nonetheless, there is a gap in the literature about the impact of exogenous progestogens at molecular level and the consequences to immature oocytes. The expression profile of genes related to the acquisition of oocyte competence, such as growth factors, maternal effect, steroidogenic pathway, apoptosis, and regulatory proteins, among others, could help in the development of more effective synchronization and/or stimulation protocols for use in assisted reproduction in vivo and in vitro.

Oocyte quality is related to developmental competence, as oocytes sustain profound changes at structural, molecular, and biochemical levels, including maternal zygotic transition. This capability is acquired during the follicular growth, mainly the final phase. However, reaching full growth does not ensure that an oocyte is capable of undergoing all the steps of embryonic development to term [16,17]. Nevertheless, studies have demonstrated that oocytes, which have already finished the exponential growth phase, screened by brilliant cresyl blue (BCB), are more competent and show a greater blastocyst rate. The BCB test measures the activity of glucose-6-phosphate dehydrogenase, which has low activity in fully grown oocytes (BCB^+) and high activity in growing oocyte ($BCB-$) [18,19]. Thus, we tested the hypothesis that P_4 and MAP promote dissimilar effects on the molecular status of COCs impacting oocyte quality. The aim of the present study was to evaluate the effect of P_4 and MAP on the gene expression profile of immature COCs and the implication of this on oocyte quality prediction in sheep.

2. Materials and methods

2.1. Local conditions and animal care

The study was performed at the *Unidade de Pesquisa Experimental em Caprinos e Ovinos*, at *Universidade Federal Fluminense*, in Cachoeiras de Macacu, Rio de Janeiro, Brazil (22°27′ S, 43°39′ W) in October of 2017 (nonbreeding season). All procedures were approved by the local Ethical Committee for Animal Use of the University (protocol #721/2015) and were conducted under the ethical principles of the Brazilian Society of Animal Experimentation. Thirty multiparous Santa Inês ewes (mean ± SD: 3.9 ± 1.0 yr old, 51 ± 5.9 kg of BW, and 3.2 ± 0.6 of BCS/scale 0–5) were used; the animals were clinically healthy, fed on chopped elephant grass (*Pennisetum purpureum*) and 200 g per animal of concentrate (12% crude protein) twice daily, and receiving water and mineralized salt ad libitum.

2.2. Experimental design

Thirty estrus-synchronized ewes were submitted to FSH ovarian stimulation at ovulation (Day 0), in the first follicular wave. At the moment of the first FSH administration, ewes were allocated into 3 experimental groups (n = 10) and received an intravaginal device containing either P_4 or MAP, or no device (control), as shown in Figure 1. Follicular aspiration was performed on Day 2 by laparoscopic ovum pick-up (LOPU), and recovered COCs were morphologically graded and tested with BCB. Selected BCB^+ COCs were used for gene expression analysis. Blood samples were collected from the jugular vein using vacutainer tubes to determine serum P_4 concentrations from Day 1 to Day 2.

2.3. Estrus synchronization

All ewes received an estrus synchronization treatment using a short-term protocol described by Balaro et al [22]. Briefly, intravaginal sponges containing 60 mg MAP (Progespon; Schering Plough Animal Health, SP, Brazil) were applied for 6 d. One day before sponge removal, 300 IU eCG (Novormon 5000; MSD Animal Health, SP, Brazil) and 0.12 mg cloprostenol sodium (Estron, Tecnopec, São Paulo, Brazil) were administered intramuscularly (i.m.). At 36 h after sponge removal, 0.025 mg lecirelin (Gestran Plus; Tecnopec, SP, Brazil) was given i.m.

2.4. Progestogen sources during ovarian stimulation

At 80 h after sponge withdrawal (defined as Day 0, ie, soon after ovulation) 80 mg of FSH (Folltropin-V; Vetoquinol, Paris, France) were administered in 3 injections (50%, 30%, and 20%) every 12 h to stimulate the first follicular wave of the estrous cycle. At the first FSH dose, the ewes received either an intravaginal device containing 0.33 mg of P_4 (CIDR, Eazi-Breed; Zoetis; P_4 group) or an intravaginal sponge containing 60 mg MAP, whereas the control group did not receive any progestogen device and remained untreated during the early luteal phase (*ie*, endogenous P_4).

2.5. Serum P_4 determinations

Blood samples were collected and centrifuged (2,600 × *g*), and serum was stored at −20°C until P_4 determination, which was performed by a solid-phase radioimmunoassay using a commercial kit (ImmuChem; MP Biomedicals, Santa

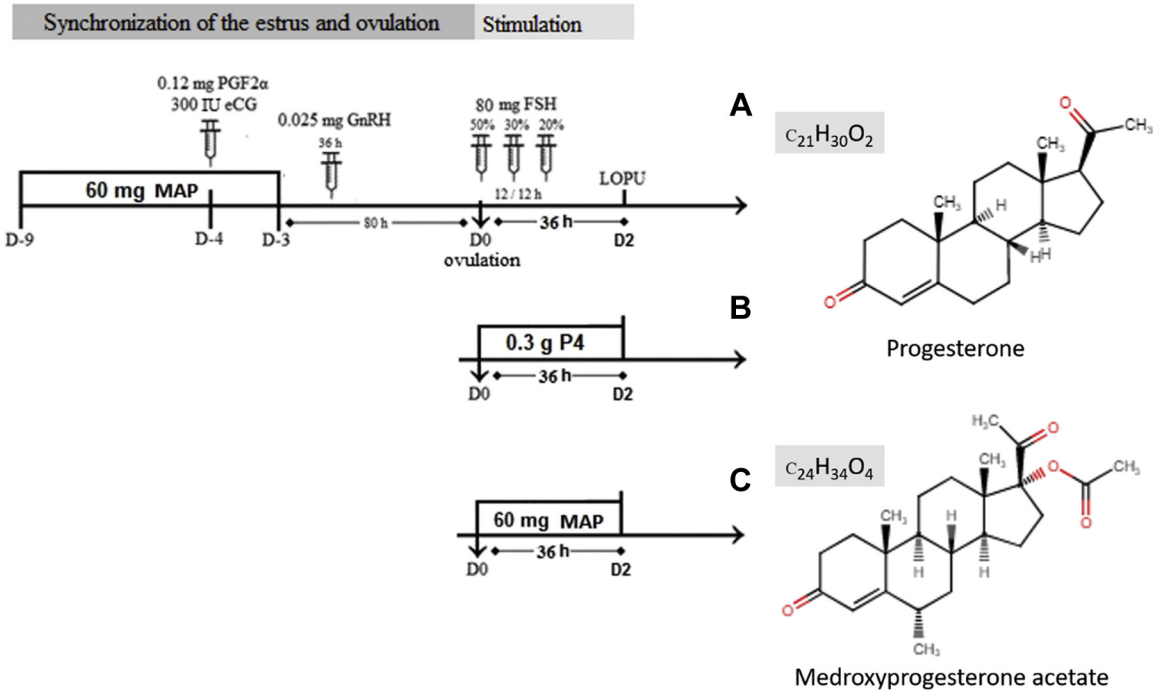

Fig. 1. Representative scheme of the experimental design. At 80 h after sponge removal of the estrus synchronization treatment (Day 0), ovarian stimulation was performed with 80 mg of FSH in 3 decreasing doses every 12 h. At first FSH dose, the females were allocated to one of 3 experimental groups: (A) No progestogen support was applied in the control group (exposed to endogenous progesterone); (B) progesterone (P_4) was administered by intravaginal devices (CIDR-G); and (C) medroxyprogesterone acetate (MAP) was administered by intravaginal devices (sponges). (A) and (B) were exposed to endogenous and endogenous plus exogenous P_4 (bioidentical, same chemical structure), respectively, while (C) was exposed to MAP synthetic progestogen analog (with a different chemical structure). Source for molecule structure [20,21].

Ana, CA, USA). The sensitivity and intra-assay coefficient were 0.05 ng/mL and 11%, respectively. All data were within the maximum and minimum point of the curve.

2.6. COCs selection and BCB test

COCs were recovered by LOPU, as previously described [8]. COCs were isolated under a stereomicroscope, and their quality was graded based on a visual assessment of morphology (number of cellular layers and cytoplasmic uniformity), with Grade I/II being good, III acceptable, and IV poor [23]. To predict developmental competence, viable COCs (GI, GII, and GIII) from each treatment were washed once and exposed to 26 μM BCB (B5388, Sigma) diluted in DMPBS supplemented with 10% BSA (A9647, Sigma) 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, placed in polyvinyl alcohol (P8136; Sigma) for 5 min, and classified according to oocyte cytoplasm staining as BCB^+ (blue cytoplasm) and BCB− (colorless cytoplasm). BCB^+ COCs from each treatment were recovered with a minimal amount of medium and transferred to DNAse/RNAse-free cryotubes, subjected to snap freezing, and stored in liquid nitrogen canisters for later gene expression analysis.

2.7. Gene expression analysis

Samples were analyzed by quantitative PCR (qPCR) after reverse transcription [24]. Total RNA was extracted from 3 pools of 5 COCs BCB^+ per group using the RNeasyMicro Kit (Qiagen Inc, Valencia, EUA) according to the manufacturer's instructions and treated with DNase for 15 min to prevent DNA contamination. Elution was performed with 14 μL of RNAase free water, and the RNA quantification of each pool was performed using 1 μL of sample on a spectrophotometer (NanoDrop 2000, Wilmington, DE, USA). For reverse transcription, using the SuperScript III first-strand synthesis Supermix (Invitrogen, Carlsbad, CA, USA), the same RNA concentration was used for all samples. The reverse transcription reaction was prepared by mixing oligo (dT) 20 primers, dNTP mixture, superscript III RT, RNase OUT, MgCl2, RT buffer, and RNA sample in a final volume of 20 μL. The mixtures were first incubated at 65°C for 5 min and then for 50°C for 50 min. The reaction was terminated at 85°C for 5 min and then chilled on ice. After that, RNase H was added to the samples and incubated at 37°C for 20 min.

Relative quantification was performed in triplicate using qPCR (ABI Prism 7300 Sequence Detection Systems, Foster City, CA, USA). Reactions (20 μL total volume) were prepared using a mixture of SYBR green kit (10 μL; Power SYBR Green, Applied Biosystems), 0.1 μM primers (Table 1), nuclease-free water, and reverse-transcribed cDNA (1 μL). Negative controls, comprising the PCR reaction mixture without nucleic acids, were also run with each group of samples. Template cDNAs were denatured at 95°C for 10 min, and all genes were amplified by 40 cycles of a thermal cycling programmed of 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

Table 1
Sequences of the specific primers used in the analysis of gene expression in immature brilliant cresyl blue–positive cumulus-oocyte complexes (BCB^+) recovered by laparoscopy after different ovarian stimulation treatments in Santa Ines ewes.

Gene	Sequence of primers (5′- 3′)	Annealing temperature (°C)	Size (bp)	Reference
GAPDH	GGGAAA TCG TGC GTG ACA TTA AG TGTGTT GGCGTAAGGTCTTTG	60	273	Bebbere et al 2008
ZAR1	CAC TGC AAG GAC TGC AAT ATC CAG GTG ATA TCC TCC ACT C	60	137	Bebbere et al 2008
MATER	CAG CCT CCA GGA GTT CTT TG GAC AGC CTA GGA GGG TTT CC	59	212	Bebbere et al 2008
GDF9	CAG ACG CCA CCT CTA CAA CA CAG GAA AGG GAA AAG AAA TGG	58	198	Bebbere et al 2008
BMP15	GGG TTC TAC GAC TCC GCT TC GGT TAC TTT CAG GCC CAT CAT	59	273	Bebbere et al 2008
BAX	CCT GGG ATC TTG AAA CTC TCC TT CTG AGC CAG GCT GAA ATC AAA A	60	566	Chakravarthi et al 2017
Bcl-2	GCC GAG TGA GCA GGA AGA C GTT AGC CAG TGC TTG CTG AGA	60	214	Chakravarthi et al 2017
ERα	GAA TCT GCC AAG GAG ACT CG CCT GAC AGC TCT TCC TCC TG	60	187	Hogg et al 2011
LHr	TCC GAA AGC TTC CAG ATG TT GAA ATC AGC GTT GTC CCA TT	60	199	Hogg et al 2011
FSHr	TAA GCA CTT GCC AGC TGT TC CTC ATC GAG TTG GGT TCC AT	60	196	Hogg et al 2011
STAR	GCA TCC TCA AAG ACC AGG AG CTT GAC ACT GGG GTT CCA CT	60	194	Hogg et al 2011
RELN	CAGCCAAAGGACTTCACCCA CGAGTGCTTACTAGCACAACC	60	161	NM_001306121.1
LRP8	ACACCTGTGGCTGTTGTTCA CCAGAGGCAGGAAAGACCTG	60	161	NM_001097565.1

Abbreviations: BAX, BCL2-associated X protein; Bcl-2, B-cell lymphoma 2; BMP15, bone morphogenetic protein 15; ERα, estrogen receptor; FSHr, FSH receptor; GAPDH, glyceraldehyde3-phosphate dehydrogenase; GDF9, growth differentiation factor 9; LHr, LH receptor; LRP-8, LDL receptor–related protein 8; MATER, maternal antigen that embryo requires; RELN, reelin; STAR, steroidogenic acute regulatory protein; ZAR1, zygote arrest 1.

PCR run, a melting curve analysis was performed to confirm that a single specific product was generated. Primer efficiency was calculated using LinRegPCR software [25] for each reaction. The primer efficiency average was 1.95; 1.96; 1.92; 1.89; 1.91; 1.93; 1.91; 1.91; 1.91; 1.93; and 1.98 to *ZAR1, MATER, BMP15*, *BAX, Bcl-2, ERα, LHr, FSHr, STAR, RELN*, and *GAPDH*, respectively. Relative quantification was performed by the comparative Ct method ($2^{-\Delta\Delta Ct}$) using the REST 2008 software [26,27]. The expression of each target gene was normalized using *GAPDH*. The groups were compared as Control vs MAP, control vs P_4, and MAP vs P_4. Fold changes are relative to the calibrator (=1), which was either the control (Control vs MAP and control vs P_4) or MAP (MAP vs P_4) data.

2.8. Statistical analysis

Variables with normal distribution were analyzed by ANOVA, and the significance of differences between mean values was determined using Tukey's test. Data presented as a frequency or percentage (ie, proportion of BCB^+ COCs) were analyzed by chi-square test. Statistical analyses were performed using a statistical analysis system program (SAEG 9.0; *Universidade Federal de Viçosa*). Differences were considered significant when $P < 0.05$. Data are given as the mean ± SEM.

3. Results

3.1. COCs recovery and gene expression

There was no effect of progestogen source on the total number of viable or BCB^+ COCs, as shown in Table 2. Gene expression was altered by exogenous progestogens compared with the control (Fig. 2A, B): Steroidogenic pathway receptors (*FSHr, LHr,* and *Erα*), as well as markers of oocyte quality (*ZAR1, GDF9*, and *Bcl-2*) genes were in abundance in P_4 ($P < 0.05$). Conversely, *RELN* was downregulated, and *Bcl-2* was upregulated in MAP ($P < 0.05$). In the comparison between the different devices, *FSHr, LHr,* and *RELN* genes were upregulated ($P < 0.05$) in P_4 compared with the MAP group.

3.2. Serum P_4 concentrations

All ewes from the 3 experimental groups had nondetectable serum P_4 concentrations 1 d before (Day −1) and on the day that treatments began (Day 0, at first FSH administration). Progesterone concentrations were affected by day and type of device ($P < 0.05$; Fig. 3), this being greater on Day 1 and Day 2 in P_4 group ($P < 0.05$), compared with the MAP and control groups. The mean concentrations on Day 2 were 1.0 ± 1.0, 0.6 ± 0.3, and 3.7 ± 0.7 ng/mL ($P < 0.05$) in the control, MAP, and P_4 group, respectively.

4. Discussion

This study provides new information about the impact of exogenous progestogens on the gene expression profile of immature COCs in the sheep model. The results demonstrate that P_4 and MAP cause dissimilar effects on gene expression in fully grown COCs (BCB^+). This effect probably occurred due to the variations in the chemical

Table 2
Effect of different progestogen treatments during FSH stimulation in the first follicular wave, on the quality (morphology and BCB test) of immature cumulus–oocyte complexes (COCs) per ewe recovered by laparoscopy in Santa Ines ewes (mean ± SE).

Group	COCs selection/ewe			
	Viable COCs[a]	Bad COCs[a]	BCB^{+}[b]	BCB^{-}[b]
P_4[c]	7.7 ± 0.7	0.5 ± 0.7	4.7 ± 1.2	3.2 ± 1.1
MAP[c]	5.7 ± 1.0	0.5 ± 0.7	3.5 ± 2.3	2.2 ± 1.5
Control[c]	5.7 ± 1.1	0.2 ± 0.4	3.6 ± 2.4	2.1 ± 1.7

Abbreviation: BCB, brilliant cresyl blue positive.
n = 10 ewes per treatment; ($P > 0.05$).
[a] Viable COCs: grading according to the cumulus cellular layers and cytoplasmic homogeneity, as good (G1, multilayered compacted cumulus and homogeneous ooplasm; G2, one to 3 layers and homogeneous ooplasm) and acceptable (G3, one incomplete layer or denuded but homogeneous ooplasm); *Bad COCs: G4 (shapeless, expanded cumulus, and degenerated) were discarded.
[b] BCB^{+}: competent fully grown COCs; BCB−: noncompetent growing COCs.
[c] P_4: Ewes received CIDR containing 0.33 mg of progesterone (P_4); MAP: ewes received sponge containing 60 mg of medroxyprogesterone acetate; Control: ewes did not receive any progestogen device.

structure of these progestogens, resulting in different activity and inducing different transcriptome profiling.

BCB test showed that oocyte exponential growth was not influenced by progestogen because there was no difference in the number of BCB^{+} or BCB^{-} COCs across the P_4, MAP, and control groups. In other words, all the groups had oocytes of similar competence prediction. The higher developmental competence of the BCB^{+} COCs compared with the BCB^{-} COCs may be related to the favorable conditions of the follicular development during the final phases of folliculogenesis and has already been demonstrated in sheep [19,28] and cows [18,29]. Therefore, only BCB^{+} COCs were used in the present study, resulting in more homogeneous samples, which enabled the attribution of differences in gene expression induced by the treatments. In addition, our recent data [5] showed no differences in follicular population and COC morphological features (GI, GII, GIII, and GIV) across the groups. Thus, FSH applied for ovarian stimulation appears to be the responsible to determine the number, growth, and morphological

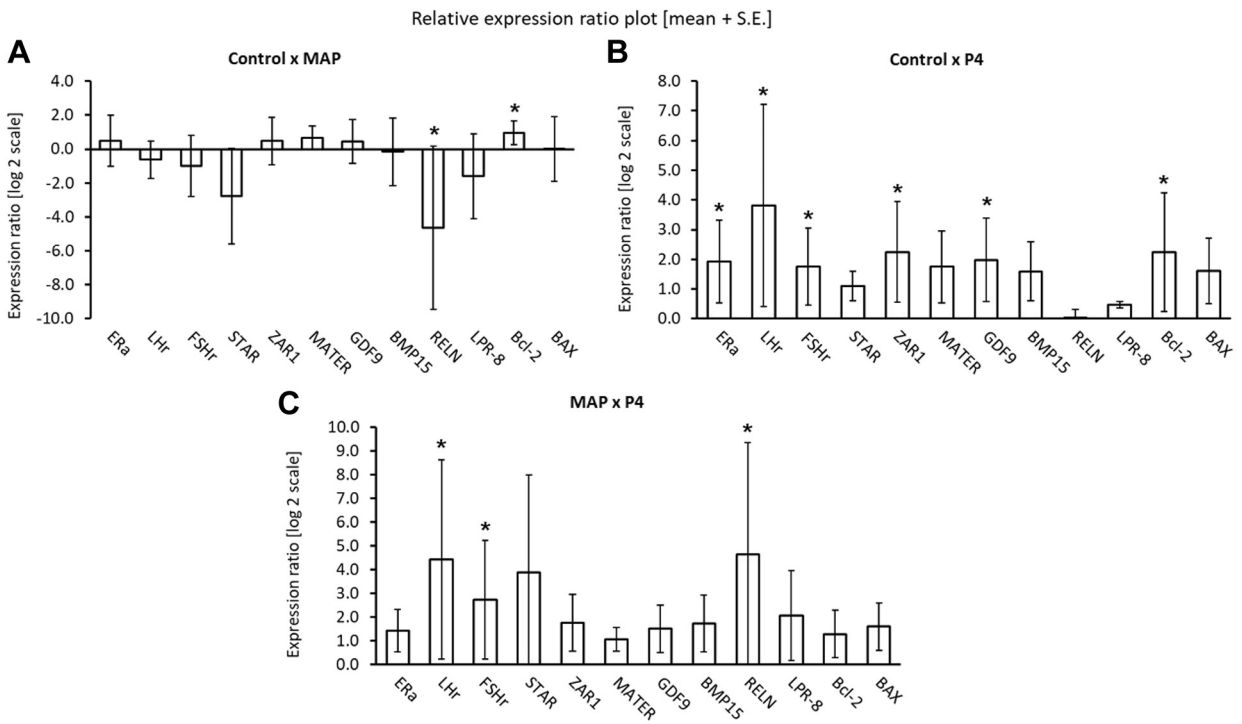

Fig. 2. Relative gene expression in immature cumulus–oocyte complexes (COCs), brilliant cresyl blue–positive (BCB+) derived from nonprogestogen-treated (control) or progestogen-treated (MAP or P4) ewes during ovarian stimulation of the follicular first wave. The groups were compared as (A) Control vs MAP, (B) Control vs P4, and (C) MAP vs P4. Fold changes are relative to the calibrator (=1), which was the control data in (A and B) and MAP data in (C). Data show means ± SE *$P < 0.05$ compared with reference gene. GAPDH, glyceraldehyde3-phosphate dehydrogenase; ERα, estrogen receptor; LHr, LH receptor; FSHr, FSH receptor; STAR, steroidogenic acute regulatory protein; ZAR1, zygote arrest 1; MATER, maternal antigen that embryo requires; GDF9, growth differentiation factor 9; BMP15, bone morphogenetic protein 15; RELN, reelin; LRP-8, LDL receptor–related protein 8; Bcl-2, B-cell lymphoma 2; BAX, BCL2-associated X protein.

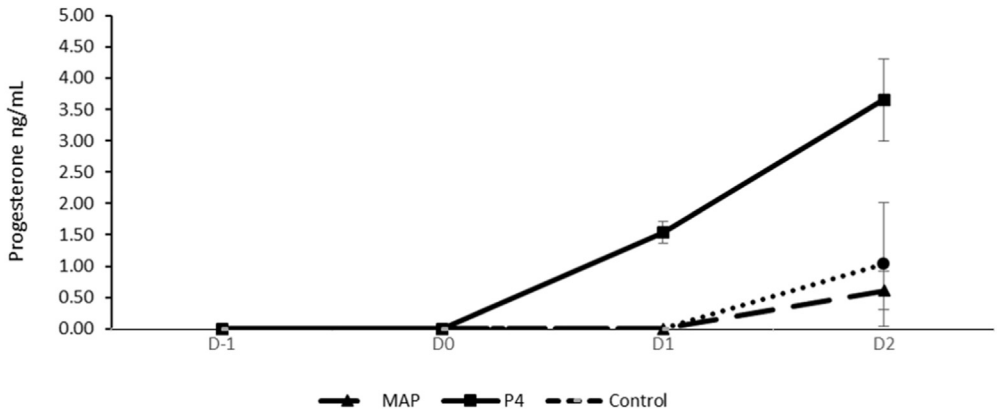

Fig. 3. Serum progesterone (P4) concentrations in Santa Ines ewes treated with progesterone (P_4 group), medroxyprogesterone acetate (MAP group), and nontreated ewes (control group) during the early luteal phase. Day 0 was defined at 80 h after sponge removal in an estrus synchronization treatment.

quality of the COCs, regardless of the progestogen used. Although progestogens had no effect on ovarian dynamics [5] and COCs morphology, P_4 and MAP promoted different effects on gene expression of COCs (BCB^+), confirming our hypothesis.

As expected, the P_4 treatment promoted greater P_4 serum concentration in comparison with MAP and control groups. Low serum P_4 in MAP and control groups was a consequence of the recent formation of the corpus luteum. These data are in agreement with Cuadro et al [13], who reported higher P_4 serum concentration in ewes receiving a P_4 implant than ewes in the early luteal phase (control) during the FSH stimulation of the first follicular wave. Similarly, Bartlewski et al [15] observed a greater P_4 concentration in exogenous P_4-treated ewes than in MAP-treated ewes during a superovulation protocol. It is well known that MAP serum concentration is not detected by the RIA kit used to measure P_4. For MAP detection, it is necessary to use a specific antiserum [30,31]. We speculate that this may occur because of the divergence in the chemical structure between MAP and P_4. The MAP molecule presents a methyl (CH_3^-) radical at C6 position and a methyl acetate ($C_2H_3O_2^-$) radical at C17 position (Fig. 1), whereas in the P_4 molecule, this radical is absent. The exogenous P_4 is bioidentical to the endogenous (luteal) hormone [11].

The abundance of messenger RNA (mRNA) encoding proteins expressed in the oocyte (ie, *MATER, ZAR1, BMP15,* and *GDF9*), cumulus (ie, *RELN, LRP8, FSHr, LHr, ERα,* and *STAR*) or both (ie, *BAX* and *Bcl-2*) were evaluated in BCB^+ COCs. In the P_4 group, 6 genes were upregulated (*ZAR1, GDF9, Bcl2, FSHr, LHr*, and *ERα*), and in the MAP group, 2 genes were affected (*RELN* was downregulated and *Bcl2* upregulated), both compared with the control (calibrator). However, comparing P_4 vs MAP (calibrator), 3 genes were upregulated (*RELN, FSHr,* and *LHr*) in the P_4 group.

The difference in *RELN* abundance (downregulated in MAP compared with control and upregulated in P_4 compared with MAP) was not related to serum P_4 concentration because *RELN* expression was no different between P_4 and control. The contrast in *RELN* mRNA expression may be due to the different chemical structure of the progestogens. We presume that P_4 and MAP pharmacokinetics activate distinct pathways, despite the similar therapeutic effect (pharmacodynamics). *RELN* expression is maximal in the theca cells of dominant follicles and binding *LRP8* in granulosa cells by paracrine action, activating the downstream signaling pathways [32], which regulate the final follicle growth stage [7]. Yang et al [33] reported *RELN* involvement with protein kinases activity and suggested it plays a role in progestogenic pathways. Conversely, Fayad et al [32] have demonstrated that *RELN* and its ligand, LRP8, are predominantly expressed in the dominant follicles and similarly downregulated during the ovulatory process and luteinization. Thus, we considered the *RELN* abundance as an important marker of oocyte competence acquisition.

We associate the gene expression profile observed in the P_4 group to the rise of serum P_4 concentrations. Recently, Menchaca et al [14] demonstrated that high serum P_4 induced by a P_4 intravaginal device enhances oocyte developmental competence by increasing the in vitro cleavage rate and embryo development in sheep (compared with nontreated ewes). Cuadro et al [13] have reported similar effects in the in vivo embryo production system in sheep, which both did and did not receive exogenous P_4 during the growing phase of the follicular wave. The authors found an improvement in the percentage of fertilized oocytes after insemination, a greater number of transferable embryos per donor, and better quality of the collected embryos. Otherwise, when P_4 priming was compared with MAP treatment, Bartlewski et al [15] reported no differences in terms of the number of corpora lutea and embryos recovered in superovulated ewes.

In support of the upregulation of *ZAR1* in the P_4-treated group in relation to the control group, we considered this a good indication of quality improvement; moreover, it may corroborate the findings of Menchaca et al [14] and Cuadro et al [13], already mentioned. *ZAR1* is a good predictor of developmental competence, mainly in immature oocytes. *ZAR1* mRNA plays a role in early embryo development until the zygotic genome activation [34,35].

FSH levels directly influence *FSHr, LHr,* and *ERα* [8,36] by downstream activation of a *FSHr*/adenylciclase/cAMP/

PKA cascade to trigger estradiol (E_2) production [37–39]. An interaction between P_4 and FSH has already been evidenced [14,40], and we suggest that this interaction could be a function of the P_4 molecular structure; it may be that the methyl acetate radical on MAP molecules may prevent this interaction. Bartlewski et al [15] observed longer FSH-metabolic clearance rates in the P_4-treated than the MPA-treated ewes. Based on this finding, and those reported by Wei et al [36] and Knecht et al [41], we speculate that the longer FSH bioavailability in P_4-treated ewes possibly maintained the stimulus of downstream FSH-induced pathways, enabling the expression of these receptors, including LHr, through raising of cAMP, despite P_4 inhibition of LH release pulsatile frequency by feedback mechanism. The upregulation of these receptors (*FSHr, LHr,* and *ERα*) may promote better conditions for oocyte to respond to the in vitro maturation conditions regarding the role of hormonal regulation during the maturation process.

The greater serum P_4 also affected *GDF9* expression, probably by extending FSH bioavailability in the P_4-treated group. Exogenous FSH affects *GDF9*, as we have previously demonstrated [8]. *GDF9* stimulates granulosa cell proliferation and acts in the regulatory process during final follicle growth, before the LH surge before ovulation; cumulus cells require *GDF9* to support the metabolic cascades and steroid production [42,43]. Hence, the P_4-treated group seems to promote more appropriate COC gene modulation.

Apoptosis proteins regulate cell death and are good markers of cell viability. The antiapoptotic *Bcl2* gene was upregulated in both the P_4- and MAP-treated groups in the present study. Similar results were obtained by Thammisiri et al [3] in a short-term MAP-treated protocol. These evidence indicate the positive effect of exogenous progestogens applied during ovarian stimulation on the apoptosis modulation cascade.

Nilsen and Brinton [44] demonstrated the divergent impact of P_4 and MAP on the mitogen-activated protein kinase (MAPK) pathway for the mechanism of neuroprotection in neuron culture, which reinforces our hypothesis about both progestogens activating different downstream pathways, despite their similar therapeutic effects. Exogenous support of P_4 seems to exert a beneficial effect on oocyte quality, based on the profile of gene expression (compared with the MAP-treated and control groups). On the other hand, MAP had a negative effect, mainly because of the downregulation of *RELN*, since this extracellular matrix glycoprotein is involved in 47 distinct cell functions, including MAPK pathway [33], which is necessary for germinal vesicle breakdown and oocyte maturation [45]. However, to confirm this deleterious effect of MAP, future research is necessary to evaluate in vitro development and molecular features of the embryos, such as polyspermy regulation, cryopreservation, and/or implantation.

In conclusion, despite P_4 and MAP promoted obtaining COCs with similar quality related to morphological features and BCB test, they induced dissimilar effects on the molecular status of immature COCs in sheep. Exogenous P_4 appears to influence indirectly the oocyte competence through its positive impact on the COC gene expression (*ZAR1, GDF9, Bcl2, FSHr, LHr,* and *ERα*), probably because of its chemical structure (identical to the P_4 endogenous) and greater serum P_4 concentration, which induce this difference in its biological activity. However, the exact mechanism by why it occurs still needs to be elucidated.

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