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Overexpression of hyaluronan synthase 2 and gonadotropin receptors in cumulus cells of goats subjected to one-shot eCG/FSH hormonal treatment for ovarian stimulation

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ABSTRACT

Hormonal ovarian stimulation may affect transcripts in somatic cells of cumulus-oocyte complexes (COCs) and affect the resulting oocyte quality. Here, in parallel with morphological classification and *in vitro* maturation (IVM) rate analysis, we investigated the expression of hyaluronan synthase 2 (*HAS2*), gonadotropic receptors (*FSHR* and *LHR*) and connexin 43 (*GJA1*) in cumulus cells (CCs) from goat COCs after multi-dose FSH (MD) or one-shot FSH/eCG (OS) treatments, using bovine COCs as control groups. The MD treatment produced more large follicles, and the resulting COCs had a better morphology and IVM rate than were obtained with OS. The OS treatment produced COCs with increased *HAS2*, *FSHR*, *LHR* and *GJA1* expression. This gene expression pattern was also observed in the CCs of COCs that showed poor morphological characteristics. On the other hand, the mRNA levels were more similar between groups after IVM; *FSHR* and *LHR* were the main genes that showed decreased expression. Some events that occurred in bovine CCs during IVM, such as cell expansion, increased *HAS2* expression and decreased *GJA1* expression, were less evident or did not occur in goat COCs. In conclusion, increasing *HAS2*, *FSHR*, *LHR* and *GJA1* expression in goat COCs does not confer greater meiotic competence to oocytes. Instead, it may result from poor regulation of gene expression in CCs by lower quality oocytes. Finally, cumulus expansion, together with *HAS2* upregulation and *GJA1* downregulation, seems to be more important for bovine COCs than for goat COCs. Additional studies are needed to investigate the importance of other HAS isoforms and connexins in goat COCs.

1. Introduction

In several ruminant species, *in vitro* embryo production (IVP) technology has become more widely used (van Wagtendonk-de Leeuw, 2006; Cox and Alfaro, 2007), especially in cattle, a species for which it has already reached commercial application (Pontes et al., 2011). However, there seems to be a consensus that *in vitro* maturation (IVM) is an important step for the success of IVP, i.e., it is necessary to produce competent mature oocytes that reach the blastocyst stage (for a review: Kane, 2003). The IVM of oocytes precedes and is required for successful *in vitro* fer-

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tilization (IVF). The cumulus cells (CCs) surrounding the immature oocyte have an important function in oocyte development. One of their functions is the channeling of metabolites and nutrients to the oocyte to help stimulate germinal vesicle breakdown and direct development to metaphase II. In fact, it has been widely reported for several species that one of the first morphological indicators of successful oocyte maturation is expansion of the cumulus mass away from the oocyte (Lonergan and Fair, 2016).

One of the principal components of the expanded cumulus-oocyte complexes (COCs) is the glycosaminoglycan (GAG) hyaluronan (HA), which is synthesized at the cell membrane by hyaluronan synthases (HAS; Weigel et al., 1997). In mammals, Watanabe and Yamaguchi (1996) found that the HAS family consists of three known isoenzymes (HAS-1 to 3). In bovine CCs, the action of the HAS2 isoform is the most important source of HA (Schoenfelder and Einspanier, 2003).

The process of cumulus expansion is accompanied by modifications of gap junctions (Pandey et al., 2009), which contain transmembrane channels formed by hexamers of proteins belonging to the connexin family (for review: Nielsen et al., 2012). Connexin 43 (alpha 1 gap junction protein of 43 kDa, GJA1) is the main protein that builds these junctions between CCs of several animal species (Santiquet et al., 2013; Li et al., 2015). Gap junction communication between an oocyte and adjacent CCs and communication between somatic cells is critical for both nuclear and cytoplasmic maturation (Vozzi et al., 2001). In equine and porcine CCs, the beginning of meiotic resumption has been associated with the reduction of the connexin 43 protein level (Marchal et al., 2003). It has long been known that during IVM of bovine COCs, the connexin 43-positive gap junctions disappear (Sutovský et al., 1993).

Gonadotropins are often added to IVM media to induce cytoplasmic maturation and CC expansion. Follicle stimulating hormone (FSH) induces the expansion of mouse COCs *in vitro* (Salustri et al., 1990) and improves bovine IVF (Izadyar et al., 1998). Luteinizing hormone (LH) has beneficial effects on bovine oocyte maturation (Zuelke and Brackett, 1990). Nonetheless, for gonadotropins to act *in vitro*, the COCs must express mRNAs and proteins encoding the FSH and LH receptors (FSHR and LHR).

In goats, information is lacking regarding the IVM process. Thus, the aim of this study was to investigate, before and after IVM, gene expression (*HAS2*, gonadotrophin receptors and connexin 43) in the CCs of goat COCs obtained by laparoscopic ovum collection after ovarian stimulation with multi-dose FSH (MD) or one-shot FSH/eCG (OS) treatments. We compared the results with those of bovine COCs.

2. Materials and methods

2.1. Animal ethics and management

All procedures in this study were performed in compliance with the Ethics Committee on Animal Use at the State University of Ceará (Number protocol 5846717/2014). A total of 14 Canindé goats aged one to three years (mean body weight ± SEM, 32.6 ± 1.92 kg) were selected as oocyte donors. All animals were maintained indoors in groups of five per pen under controlled nutrition. They had access to a Tifton (*Cynodon dactylon*) pasture in the morning and received Tifton during the afternoon. Additionally, goats were supplemented with good-quality concentrate (20% crude protein) and had free access to water and minerals. Bovine ovaries, goat ovaries and goat brain tissues were obtained from a local slaughterhouse.

2.2. Hormone treatments

The animals were subjected to two ovarian stimulation protocols. Each hormonal treatment was performed in four sessions with seven animals per session per protocol. The estrous cycles of all oocyte donors were synchronized using intravaginal sponges impregnated with medroxyprogesterone acetate (60 mg; Progespon; Syntex, Buenos Aires, Argentina), which were inserted for 10 days, combined with an intramuscular (im) luteolytic injection of cloprostenol (50 μg; Ciosin; Coopers, São Paulo, Brazil) on the 7th day of progestagen treatment. The ovarian stimulation consisted of two experimental groups: the standard ovarian stimulation was carried out using multiple doses (MD) of pFSH (120 mg; Folltropin-V; Bioniche, Belleville, Canadá) distributed across five injections (30/30; 20/20; 20 mg) at 12 h intervals starting on the 7th day of progestagen treatment. The one-shot treatment (OS) used pFSH (70 mg) and a single dose of eCG (200 IU; Novormon; Syntex, Buenos Aires, Argentina) administered 36 h before sponge removal.

2.3. Cumulus-oocyte complex recovery

Follicle aspiration for oocyte harvest was performed through the laparoscopic method, as previously described by Baldassarre et al. (2003). In brief, goats were fasted for 36 h from food and water prior to oocyte recovery by laparoscopy (LOR). The surgery was performed under general anesthesia, starting with an intravenous anesthetic induction using thiopental at a dose of 20 mg/kg (Tiopentax 2.5%; Cristália, São Paulo, Brazil). Deep anesthesia was maintained with 3% isoflurane (Isoforine, Cristália, São Paulo, Brazil) with the help of inhalation anesthesia equipment (HB Hospital, São Paulo, Brazil). The COCs were aspirated from follicles >2 mm in diameter that were visible on the ovary's surface using a 22-gauge needle (Watanabe, São Paulo, Brazil) and a vacuum pump (WTA, Cravinhos, São Paulo, Brazil). The vacuum pressure was regulated at 35 mmHg. The collection medium used was TCM199 (Nutricell, Campinas, Brazil) buffered with 10 mM HEPES and supplemented with 20 UI/mL heparin, 0.2 mM pyruvate, 100 U penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin (Sigma-Aldrich, St. Louis, USA), and 10% fetal bovine serum (Life Technologies, New York, USA).

Bovine ovaries were transported from the slaughterhouse to the laboratory in a warmed (30–35 °C) 0.9% NaCl solution supplemented with 40 μg/mL gentamicin (Sigma-Aldrich, St. Louis, USA). The COCs were aspirated gently from follicles using an 18-gauge needle attached to a 10-mL syringe and recovered in the same collection medium used for goats.

2.4. COC grading, IVM and sampling

The COCs from each ovarian stimulation procedure were selected under a stereomicroscope and graded from GI to GIV based on cellular layers and cytoplasmic uniformity, as described by Almeida et al. (2010). Thus, the GI COCs were multilayered, compact CCs with a finely granulated oocyte cytoplasm. The GII structures showed one to three layers of CCs and a finely granulated oocyte cytoplasm. The GIII COCs showed incomplete or no CCs or a heterogeneous oocyte cytoplasm. The GIV COCs showed abnormal shapes and a heterogeneous oocyte cytoplasm or apoptotic oocytes in a jelly like cumulus-corona cell vestment.

For IVM, COCs were pooled into two groups (GI/GII and GIII) from each stimulation protocol, and they were washed and incubated in maturation medium (500 μL) in four-well plates. The medium used for the maturation of goat COCs was TCM199 (Nutricell, Campinas, Brazil) buffered with 10 mM HEPES, which contained 10 ng/mL EGF, 100 μM cysteamine, 0.2 mM pyruvate, 1 μg/mL estradiol, 100 U penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin (Sigma-Aldrich, St. Louis, USA), 20 μg/mL FSH/LH (Calier laboratories, Barcelona, Spain) and 10% goat serum from goats in estrus. Bovine structures were matured in a similar medium, with replacement of the goat serum in estrus by 10% fetal bovine serum (Life Technologies, New York, USA). The IVM conditions were 5% CO_2 in humidified air at 38.5 °C for 24 h.

After maturation, CCs were removed from each individual oocyte by repeatedly pipetting in the presence of 10 mg/mL hyaluronidase (Sigma-Aldrich, St. Louis, USA). The oocytes were stained with Hoechst 33342 (15 μg/mL) (Sigma-Aldrich, St. Louis, USA) and evaluated on an inverted microscope (TE2000, Nikon, Tokyo, Japan). Oocytes showing the first polar body and the metaphase plate with no apparent sign of degeneration were considered mature (MII). Oocytes without visible polar bodies were classified as non-competent. Oocytes subjected to the same steps but not to IVM were considered to be at the germinal vesicle stage (GV). The CCs from MII and GV oocytes were used for RNA extraction. CCs from non-competent oocytes were discarded.

2.5. Collection of experimental samples

The CCs of GI/GII and GIII COCs containing immature (GV) or mature (MII) oocytes were collected separately for real-time PCR analysis as described by Almeida et al. (2010). Briefly, CCs from 30 graded COCs (GI/II or GIII) and classified oocyte (GV or MII) were pooled separately in individual tubes, quickly spun, snap-frozen with 25 μL of RLT buffer and stored at −80 °C until RNA extraction. In all of the goat groups, COCs were obtained from at least three sessions of ovarian stimulation/laparoscopic recovery. Groups of bovine CCs were prepared by the same way and were obtained from at least three slaughter sessions with 10 animals each.

2.6. Total RNA extraction

After the culture medium was removed from the CCs by washing with PBS (made with DEPC-water), total RNA was extracted using the RNeasy micro kit (Qiagen Inc., Valencia, USA) according to the manufacturer's instructions. In brief, each frozen sample was resuspended in 50 μL of RLT Plus buffer and mixed with an equal volume of 70% ethanol. The mixture was then transferred to an RNeasy MinElute spin column from the RNeasy Micro kit for RNA to bind to the column. The RNA purification included an RNAse-free DNase treatment for 15 min at room temperature. After three washes, the RNA was eluted with 12 μL of RNAse-free water.

Tissues and cells known to express each target gene were also used as positive controls. Thus, because connexin 43 has been described in brain cells (Wu et al., 2015), total RNA was extracted from goat brain samples using Trizol reagent (Invitrogen, Califórnia, USA) following the manufacturer's instructions. Additionally, RNA samples from bovine CCs and the goat ovarian cortex were used as positive controls for *HAS2* (Kimura et al., 2002) and *FSH*/*LH* (Nuttinck et al., 2004) expression, respectively. Then, RNA samples from bovine CCs were prepared using the RNeasy micro kit as described above for goats. Additionally, RNA from the goat ovarian cortex was obtained by using the ReliaPrep RNA Tissue Miniprep System (Promega, Madison, USA) according to the manufacturer's instructions. For all samples, RNA quantification was performed using a Qubit 2.0 Fluorometer and Quant-iT RNA assay kit (Invitrogen, Califórnia, USA).

2.7. Reverse transcription

Reverse transcription was performed by adding the following to each RNA sample: oligo-dT primer (20 μM; Promega, Madison, USA); 1 μL of Improm II (1 μL; Promega, Madison, USA); dNTPs (0.5 mM of each; Promega, Madison, USA); Recombinant RNasin (2 U/μL; Promega, Madison, USA); and RNase-free water for a final reaction volume of 20 μL. Reactions were heated to 70 °C for 5 min and then placed on ice. Reverse transcription was performed at 42 °C for 60 min and then at 70 °C for 15 min. The first strand cDNA products were then stored at −80 °C for later use as templates for real-time PCR. Negative controls and RT blanks were prepared under the same conditions, but without the inclusion of reverse transcriptase.

2.8. Real-time PCR

Specific primers for the amplification of each gene (Table 1) were designed using Primer-BLAST (Ye et al., 2012) and by multiple sequence alignment of goat and bovine sequences using ClustalW2 (Larkin et al., 2007). Relative quantification was performed in triplicate for the *GJA1*, *HAS2*, *FSHR*, *LHR*, *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase), *UXT* (ubiquitously-expressed, prefoldin-like chaperone) and *H2AFZ* (member Z of H2A histone family) genes using a MasterCycler ep Realplex 4S (Eppendorf, Hamburg, Germany). Reactions (15 μL total volume) contained the following: 2× Fast SYBR Green Mas-

Table 1
Primers used in quantitative real-time PCR of gene expression in goat and bovine cumulus cells.

Gene	Primer	Nucleotide sequence (5′-3′)	Product size (bp)	GenBank
GJA1	SE	AAGCTTTGTAGGCCTGACCC	94	XM_005684517.1
	AS	TGTGGGCCTTGTCTGAACTC		
HAS2	SE	GCTGAGTCTGGGCTATGCAA	241	XM_005688873.1
	AS	ACCCCTGTAGAAGAGCTGGAT		
FSHR	SE	ATCAACTCCTGTGCCAACCC	105	XM_010809895.1
	AS	GGCTTGCACTTCATAGCAGC		
LHR	SE	GATTTCACCTGCATGGCACC	130	XM_005686598.1
	AS	ACGGATTGGCGCATGAATTG		
GAPDH[a]	SE-1	TTCAACGGCACAGTCAAGG	119	NM_001034034
	AS-1	ACATACTCAGCACCAGCATCAC	120	AJ431207.1
	SE-2	TTCCACGGCACAGTCAAGG		
	AS-2	ACGTACTCAGCACCAGCATCAC		
UXT	SE	GCGCTACGAGGCTTTCATCT	129	XM_005700842.1
	AS	TCCTGGAGTCGCTCAATGAC		
H2AFZ	SE	TCCGGAAAGGCCAAGACAAA	81	XM_005681391.1
	AS	GTGTCGATGAATACGGCCCA		

[a] Specific primers were designed to amplify the bovine (SE-1 and AS-1) and goat (SE-2 and AS-2) *GAPDH* genes.

ter Mix (7.5 μL; Applied Biosystems, California, USA), each primer (0.3 μM for *GAPDH*, *H2AFZ*, *FSHR*, and *LHR*, and 0.6 μM for *UXT* and *GJA1*, and 1.0 μM for *HAS2*) and cDNA (2–2.5 ng of RNA, corresponding to the amount of cells around one COC). Template cDNAs were denatured at 95 °C for 2 min followed by 40 cycles of an amplification program of 95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s. Fluorescence data were acquired during the 72 °C extension steps. The specificity of each reaction was ascertained after completion of the amplification protocol. After each PCR run, a melting curve analysis was performed for each sample to confirm that a single, specific product was generated.

2.9. Data and statistical analysis

GraphPad InStat 3.10 (GraphPad Software, Inc., La Jolla, USA) was used for all statistical analysis. The percentages of follicle size, graded COCs and maturations were analyzed using Fisher's exact test. The relative quantification of the gene expression was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Target gene expression was normalized against the geometric mean of the *GAPDH*, *H2AFZ* and *UXT* transcript levels. The threshold and threshold cycle (Ct) values were automatically determined by Realplex 2.2 (Eppendorf, Hamburg, Germany) using the default parameters. Expression profiles were presented as the mean (±SD) of relative mRNA abundance and were compared using one-way ANOVA followed by a Tukey test and paired *t*-test. The mRNA abundance in the CCs was the dependent variable and the fixed effects were the CCOs morphological grade (GI/GII vs GIII) or the hormonal treatment (MD vs OS). Additionally, the mRNA abundance in the CCs was also verified according to the nuclear maturation stage of the oocyte (GV vs MII) or hormonal treatment (MD vs OS). No comparisons between species (goat vs bovine) were made. The melting temperature (Tm) data were expressed as the mean (±SD) of three measurements. A value of $P < 0.05$ was set as the limit for statistical significance.

3. Results

3.1. COC collection and IVM

Qualitative and quantitative aspects of COCs produced by the two hormone treatments were compared (Table 2). At the end of the four hormonal sessions for the MD treatment, 307 punctured follicle yielded 231 COCs, resulting in a recovery rate of 75.2% (231/307). In the OS treatment, 172 COCs were obtained from 237 aspired follicles, representing a recovery rate of 72.6% (172/237). The MD treatment produced a greater amount ($P < 0.05$) of COCs with good morphology (77.5% GI/II) than the OS treatment (50.0% GI/II). The COCs from the MD treatment had a greater maturation rate than the OS treatment (81.8% vs. 63.6%, $P < 0.05$).

3.2. Gene expression in graded COCs

Initially, validation of the qPCR amplification conditions was performed for each gene (*GJA1*, *FSHR*, *LHR*, *HAS2*, *GAPDH*, *H2AFZ* and *UXT*) by constructing standard curves based on serial dilutions of cDNAs from different cells and tissues. Thus, the amplification parameters, such as the efficiency and linearity (Table 3), ensured that the qPCR conditions were suitable for subsequent quantitative analysis.

Regarding *HAS2* expression (Fig. 1), goat CCs derived from the OS treatment had greater transcript abundance ($P < 0.05$) compared to the MD group, regardless of the grading in morphological classification. The GI/II CCs showed more *HAS2* expression than that of GIII ($P < 0.05$) in both goat and bovine species.

For FSHR, bovine GI/II CCs (Fig. 1) showed greater expression ($P < 0.05$) than goat CCs (from MD or OS). In addition, CCs from the OS group had more transcripts than CCs from the MD group ($P < 0.05$). For the grade III structures, receptor expression was similar between MD and OS ($P > 0.05$). Additionally, CCs of GI/II structures showed increased *FSHR* mRNA levels ($P < 0.05$) compared to the GIII CCs for OS and bovine structures, but not for MD.

Table 2
Number of collected and graded cumulus-oocyte complexes (COCs) and the *in vitro* maturation (IVM) rates in goats hormonally treated for ovarian stimulation.

Hormonal treatment	Aspirated follicles	Follicle size (%)			Collected COCs	Graded COCs (%)		IVM rate (%)
		Small(2–3 mm)	Medium(3–4 mm)	Large(>4 mm)		GI/II	GIII	
MD	307	42.6[a]	32.6[a]	24.8[a]	231	77.5[a]	15.1[a]	81.8[a]
OS	237	61.2[b]	21.1[a]	17.7[b]	172[*]	50.0[b]	27.3[b]	63.6[b]

Values with different letters (a,b) in the same column are significantly different ($P < 0.05$).
[*] Some COCs from OS treatment showed a slight expansion of CCs immediately after laparoscopic recovery.

Table 3
Quantitative RT-PCR amplification parameters for gene expression analysis in goat and bovine cumulus cells.

Gene	Slope	Y-Intercept	Efficiency	R^2	cDNA[a]	Mean Tm (°C) ± SD
GJA1	−3.247	23.08	1.03	0.996	Goat CNS	76 ± 0.3
FSHR	−3.269	27.22	1.02	0.991	Goat ovary	79 ± 0.37
LHR	−3.379	26.89	0.98	0.990	Goat ovary	78.3 ± 0.27
HAS2	−3.298	21.91	1.01	0.993	Bovine CCs	79.8 ± 0.36
GAPDH	−3.276	20.93	1.02	0.978	Bovine embryo	82.7 ± 0.26
	−3.447	23.26	0.95	0.985	Goat COCs	81.7 ± 0.10
H2AFZ	−3.283	22.21	1.02	0.977	Bovine embryo	83.9 ± 0.27
UXT	−3.260	26.91	1.03	0.996	Goat CNS	81.3 ± 0.3

[a] Positive controls were obtained from tissues or cells known to abundantly express each target gene.

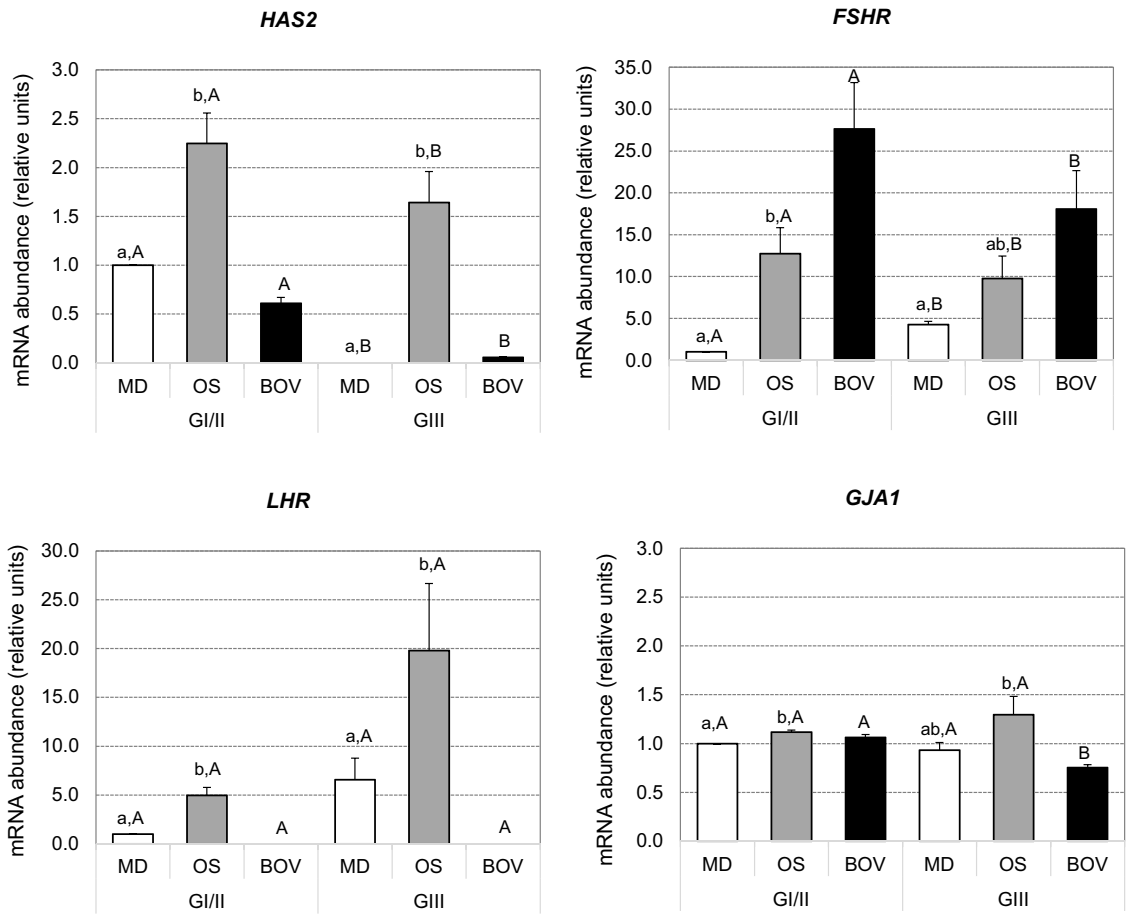

Fig. 1. Expression of *HAS2*, *GJA1*, *FSHR* and *LHR* in caprine and bovine cumulus cells from graded cumulus-oocyte complexes (COCs). Goat COCs were obtained after multidose (MD) or one-shot (OS) *in vivo* FSH treatment for ovarian stimulation. Shown are the fold differences in mRNA expression in the in GI/II and GIII COCS after normalization to the reference genes (*GAPDH*, *H2AFZ* and *UXT*). The mRNA abundance in GI/II of goat MD cumulus cells were arbitrarily set to one-fold. Morphological grades were compared within the goat hormonal treatments or for cattle as a group (A,B: $P < 0.05$). The goat (MD or OS) groups were compared within the same grade (a,b: $P < 0.05$). Bovine groups were not compared with goats.

Transcripts for *LHR* (Fig. 1) were more abundant in CCs from the OS treatment than in cells from the MD treatment ($P < 0.05$), independent of the morphological grade. However, when comparing GI/II and GIII structures, the abundance of the *LHR* transcripts were equivalent ($P > 0.05$) for each group.

The mRNA abundance for *GJA1* (Fig. 1) were greater ($P < 0.05$) in the GI/II CCs obtained from OS treatment in comparison to MD. With respect to the GIII structures, the CCs of the MD group showed similar expression levels to the OS ($P > 0.05$). The mRNA levels of this connexin in goat CCs were also similar ($P > 0.05$) between GI/II and GIII COCs. However, bovine CCs showed more *GJA1* transcripts in GI/II than in GIII structures ($P < 0.05$).

3.3. Gene expression in pre- and post-IVM COCs

The mRNA levels were also compared between pre- and post-IVM COCs, which contained GV and MII oocytes, respectively. Thus, the HAS2 mRNA levels strongly increased in bovine CCs ($P < 0.05$) after IVM. However, the transcript levels slightly diminished in goat CCs following OS treatment (MII vs GV, $P < 0.05$) and did not change in CCs following MD treatment (MII vs GV, $P > 0.05$) during IVM. In addition, *HAS2* transcripts were similar between MD and OS post-IVM COCs ($P > 0.05$).

Lower abundance of *FSHR* transcripts were obtained in CCs of MII oocytes compared to GV ($P < 0.05$), except for the structures obtained from MD treatment ($P > 0.05$). In mature COCs, FSHR expression in CCs was similar between goat hormonal treatments ($P > 0.05$).

The IVM process also reduced the *LHR* expression ($P < 0.05$) in CCs from both the OS and MD groups, but it did not change in bovine CCs, which remained null. Additionally, comparing only CCs of MII oocytes, the *LHR* mRNA level was slightly higher in the MD group than in the OS group ($P < 0.05$).

The expression of *GJA1* (Fig. 2) was similar between the MII- and GV-stage goat groups ($P > 0.05$). In the bovine group, the CCs of the MII oocytes had slightly lower expression ($P < 0.05$) than the CCs of the GV structures. Finally, in post-IVM COCs, goat CCs from OS hormonal treatment showed *GJA1* expression at the same abundance than cells from MD group ($P > 0.05$).

4. Discussion

In the present study, we analyzed the influence of gonadotropin-based ovarian stimulation on the expression of HAS2, gonadotropin receptors (FSHR and LHR) and connexin 43 in goat CCs using bovine COCs as control groups. Additionally, we analyzed transcript abundance in CCs with respect to the morphology of COCs (GI/II or GIII COCs) and the nuclear maturation stage of the oocyte (GV or MII).

The production of hyaluronan (HA) occurs at the end of folliculogenesis and is increased by gonadotropic hormone stimulus (Kimura et al., 2002). In CCs, HA is synthesized by the enzymatic action of hyaluronan synthase, HAS (Sutton-McDowall et al., 2010), in glucose metabolism. Although there are three HAS isoenzymes (HAS-1, -2 and -3) that differ in their ability to synthesize HA (Dicker et al., 2014), HAS2 has been found to be the main enzyme responsible for the synthesis of HA in bovine CCs (Schoenfelder and Einspanier, 2003).

We found greater expression of *HAS2* after OS treatment compared to the MD group; this was true in both the GI/II and GIII structures. Additionally, freshly collected COCs, despite being in the GV stage, showed slightly expanded CC populations. Previous studies have attributed the increased HA synthesis in CCs through *in vitro* expansion of COCs to the actions of gonadotropin (Schoenfelder and Einspanier, 2003). Kimura et al. (2002) observed a great expansion of porcine CCs produced by the synergistic effect of eCG with the follicular fluid of COCs cultured *in vitro*. Our findings indicate a possible *in vivo* action of eCG in OS treatment; eCG stimulated HA synthesis in the CCs of freshly collected COCs. Similar results have already been described in swine and mice. Thus, Kawashima et al. (2008) observed an increase in the *HAS2* expression of CCs from COCs derived from sows treated with eCG or eCG/hCG compared to the untreated group. Similarly, the hormonal ovarian stimulation of mice with eCG/hCG resulted in COCs with greater levels of *HAS2* mRNA (Fulop et al., 1997; Adriaenssens et al., 2011). In women treated with hCG, Jeppesen et al. (2012) reported that the cumulus of pre-IVM COCs presented a compact or expanded morphology immediately after isolation. Although the authors did not investigate HAS expression, we hypothesize that the slight expansion observed in goat COCs derived from OS treatment was produced by increased HA synthesis after *in vivo* eCG action, which did not occur in the MD protocol.

During follicular development, there are gonadotropin-dependent stages in which FSH and LH can act on receptors (FSHR and LHR) in the somatic cells surrounding the oocyte (Byskov et al., 1997; Mihm and Austin, 2002). We produced quantitative and qualitative variations in an exogenous source of gonadotropins (FSH and eCG) through two hormonal protocols for goat ovarian stimulation (MD and OS). Thereafter, we investigated the expression of *FSHR* and *LHR* in CCs of the obtained COCs.

The CCs of goat COCs obtained after OS treatment showed greater expression of *FSHR* than after the MD treatment, especially in the GI/II structures. Despite the lack of studies on the *in vivo* effects of eCG (or eCG/FSH) on the expression of FSH receptors in goats, it is possible that the *in vivo* stimulation by this hormone in the OS group caused the increased expression of *FSHR*. This *in vivo* effect was previously reported in rats treated with eCG in which there was an increase in FSHR expression in CCs, as revealed by immunohistochemistry of the ovary (Guan et al., 2015).

The goat structures derived from the OS treatment showed greater *LHR* expression than those derived from MD treatment. In pre-ovulatory follicles, *LHR* expression has been described in granulosa cells (Eppig et al., 1997; Marsters et al., 2015) and was found to be low or undetectable in CCs (Jeppesen et al., 2012). Thus, a greater level of *LHR* in OS CCs is an unexpected finding, which we believe to be related to the lower quality of the COCs. A study by Vigone et al. (2015) using mice showed that analysis of *LHR* expression by real-time qPCR can differentiate between CCs associated with competent or incompetent *in vitro* development of an oocyte. Hence, *LHR* was increased in

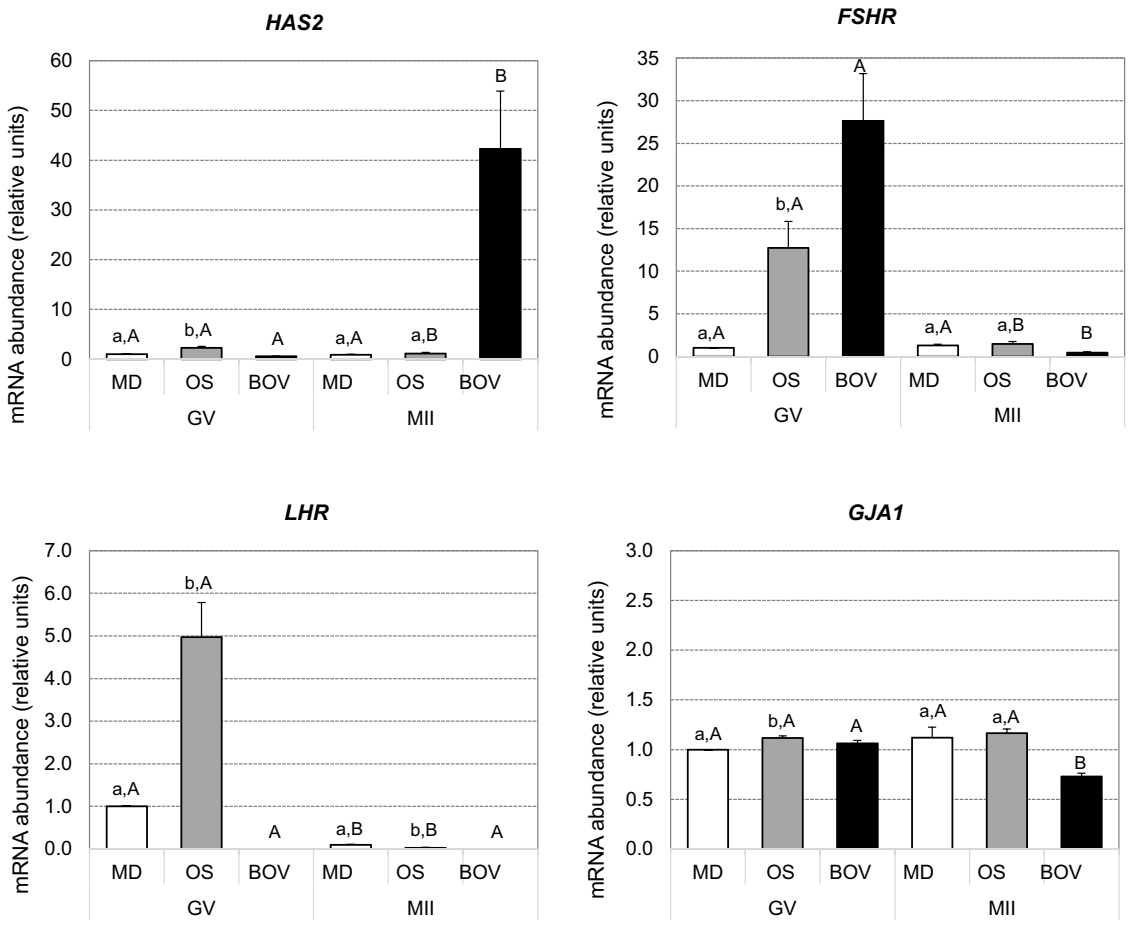

Fig. 2. Expression of *HAS2*, *GJA1*, *FSHR* and *LHR* in caprine and bovine cumulus cells from *in vitro* pre-maturation and post-maturation cumulus-oocyte complexes (COCs). Goat COCs were obtained after multidose (MD) or one-shot (OS) *in vivo* FSH treatment for ovarian stimulation. Cumulus cells were derived from pre-IVM or post-IVM COCs with germinal vesicle (GV) or metaphase II (MII) oocytes, respectively. Shown are the fold differences in mRNA expression after normalization to the reference genes (*GAPDH*, *H2AFZ* and *UXT*). The mRNA abundance in GV of goat MD cumulus cells were arbitrarily set to one-fold. Pre-IVM and post-IVM stages were compared within the goat hormonal treatments or for cattle as a group (A,B: $P < 0.05$). The goat (MD or OS) groups were compared separately for the pre-IVM and post-IVM stages (a,b: $P < 0.05$). Bovine groups were not compared with goats.

the CCs of COCs with poor-quality oocytes. Similarly, Eppig et al. (1997) reported that murine CCs only upregulated *LHR* transcripts in the absence of oocytes. In addition, *LHR* transcription was prevented by paracrine factors secreted by isolated oocytes. Thus, it is possible that poor-quality oocytes failed to provide signaling to maintain low *LHR* levels in the CCs of terminal antral follicles. This statement is also compatible with the low IVM rates that we observed in structures from OS treatment and COCs displaying the worst morphology (GIII). Finally, the difference in *LHR* abundance among post-IVM structures was absent because gene expression measurements were restricted to the CCs of successfully matured oocytes. During IVM, the COCs showed reduced *LHR* abundance in CCs, irrespective of the hormonal treatment used before COL, and *LHR* remained absent in bovine CCs.

The expansion of cumulus cells appears to be essential for cytoplasmic oocyte maturation and requires the synthesis and organization of an extracellular matrix, of which HA is the main component (Chen et al., 1994; Schoenfelder and Einspanier, 2003). Therefore, we analyzed whether IVM could influence the expression of *HAS2* in goat CCs. In the CCs of cattle, we observed that in post-IVM structures, the expression of this enzyme is much greater than in pre-IVM COCs. Conversely, the mRNA abundance were maintained (or reduced) during IVM in goat structures (MD and OS). These findings corroborate previous reports on the importance of the expansion of CCs in bovine IVM (Caixeta et al., 2013). This phenomenon is less morphologically evident in goats than in bovine IVM COCs. Hence, we hypothesized that bovine COCs have a greater abundance for cumulus expansion than goats due to higher levels of *HAS2* transcripts and greater accumulation of extracellular matrix during the IVM process. It is possible that these phenomena have different degrees of importance for IVM depending on the animal species. Thus, as we observed in goats, *HAS2* expression in sheep COCs was similar before and after IVM (Kyasari et al., 2012). Possibly, the synthesis of HA and expansion of CCs is not as important in goat and sheep IVM as it is in cattle. It is important to note that despite *HAS2* expression, the goat IVM rates had the expected val-

ues based on previous reports for this species (Khatun et al., 2011; Abdullah et al., 2008).

The FSHR in the CCs plays an important role in triggering the physiological mechanisms involved in IVM in species, such as swine and cattle (Liu et al., 1998; Nuttinck et al., 2004). In cattle, FSH and other factors secreted by the oocyte are responsible for events such as CCs expansion, increased *HAS2* expression (Gui and Joyce, 2005) and meiotic resumption (Ali and Sirard, 2005). In our experiments, we observed that *FSHR* expression decreased in CCs during IVM of both goats and cattle COCs. Our findings are similar to various reports for animals, such as cattle (Nuttinck et al., 2004; Salhab et al., 2011; Khan et al., 2015) and buffalo (Pandey et al., 2010). These findings make clear the importance of this gonadotropin receptor in the process of maturation and the loss of its function in subsequent steps of oocyte *in vitro* development.

Some studies have reported the roles of gonadotropic hormones, especially FSH and LH, in regulating the expression of connexin 43 in ovarian follicular cells (Sommersberg et al., 2000; Granot and Dekel, 2002; Johnson et al., 2002). Connexin 43 is the main protein that builds gap junctions between CCs of COCs in several animal species (Dell'Aquila et al., 2004; Pant et al., 2005; Wang et al., 2009; Santiquet et al., 2013; Li et al., 2015). These cellular communications play a crucial role in folliculogenesis and oocyte maturation (Ackert et al., 2001; Luciano et al., 2004; El-Hayek and Clarke, 2015). Thus, we investigated the influence of gonadotropic OS and MD treatments on *GJA1* expression in goat CCs and investigated possible changes in the transcript levels caused by IVM. Cattle were used as a control group.

Regarding the effects of gonadotropin *in vivo*, we found that, in general, *GJA1* expression was slightly greater in goat structures derived from OS treatment than those from MD treatment. Evidence for stimulatory FSH (Sommersberg et al., 2000; Wang et al., 2013) and inhibitory LH (Granot and Dekel, 1994; Kalma et al., 2004) effects on the abundance of *GJA1* in granulosa ovarian cells of some species have been reported. Interestingly, in our study, eCG/FSH (OS treatment) was more effective in inducing *GJA1* expression in goat CCs than FSH alone in MD. Because eCG in non-equine mammals exerts biological activity in both FSH and LH receptors (Murphy and Martinuk, 1991), it is relatively difficult to dissociate the effects of these two receptors in OS treatment. More intense or lasting action of eCG on FSHR could explain the greater abundance of connexin transcripts in OS structures. This statement is consistent with the widespread use of eCG, which is replacing FSH multiple-dose treatment as an alternate protocol for ovarian stimulation (Sousa et al., 2011). Another possibility is that the *in vivo* gonadotropic action varies the expression of connexin according to follicular size. Thus, Johnson et al. (2002) proposed that FSH promotes an increase in the number of gap junctions in granulosa cells obtained from small follicles, but not in medium or large follicles in cattle. Taking into account that more than half of COCs following OS treatment are recovered from small follicles (2–3 mm), *in vivo* gonadotropic stimulation and the resulting *GJA1* expression may have favored this group compared to MD.

When we analyzed connexin expression according to the IVM process, we found that the *GJA1* mRNA abundance slightly decreased only in bovine structures, but not in goat CCs. Some studies conducted in pigs and horses have pointed to an important role played by connexins in the maintenance of meiotic arrest of the oocyte (Marchal et al., 2003). The downregulation of connexin or rupturing of gap junctions may have been associated with meiotic resumption because they prevented inhibitory signals from passing from CCs to oocytes (Isobe et al., 1998). Our findings for cattle are in agreement with previously described results, in that the IVM process caused a decrease in *GJA1* transcript abundance in COCs (Sutovský et al., 1993). We do not know if the gap junctions play a similar role in goat COC IVM because the *GJA1* abundance remained unchanged in CCs during this process, although oocyte nuclear maturation was completed. Though it is unlikely, a connexin other than GJA1 could be involved in gap junctions in goat CCs.

Finally, we also investigated the influence of hormonal treatments on the quantitative yields of COCs for IVM and measured meiotic competence. The results showed that MD treatment produced greater amounts of large follicles and GI/II COCs than the OS protocol. Moreover, oocytes from MD COCs showed improved meiotic competence, reflected by a greater IVM rate. In previous studies (Almeida et al., 2010), we compared MD treatment with another treatment consisting of three doses (TD) of FSH. Despite obtaining similar quantities of GI/II COCs, the IVM rate was greater in the MD treatment than in TD. In sum, we concluded that ovarian stimulation of goats with multiple FSH doses produces a greater number of follicles available for LOR and produces structures that are suitable for IVM (GI/II). This explains the improved IVM rates compared to a low number of FSH administrations or treatment with a single dose of FSH/eCG.

In conclusion, somatic cells of goat COCs produced after hormonal treatment for ovarian stimulation with a single dose of FSH/eCG have greater abundance of *HAS2*, *FSHR*, *LHR* and *GJA1* compared to conventional multiple-dose FSH treatment. These transcription patterns did not confer greater meiotic competence to the oocytes. Instead, they may result from the poor regulation of gene expression in low-quality oocytes. Finally, some events that occurred in CCs during IVM of bovine COCs, such as cell expansion, increased *HAS2* expression and decreased *GJA1* expression, seem to be less evident in goat COCs. Additional studies are still needed to investigate whether other isoforms of hyaluronan synthase or connexin are more important for goat COCs, which could reflect species-specific differences in molecular biology.

Conflict of interest

The authors declare no conflicts of interest.

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