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FSH and LH enhance the development of goat preantral follicles cultured *in vitro*

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Abstract

This study evaluated the effect of increased follicle stimulating hormone (FSH) concentrations on the expression of mRNA for LH receptors after *in vitro* culture of goat preantral follicles (≥150 μm) for 18 days. It also investigated whether the addition of luteinizing hormone (LH) to the culture medium, which contained increasing concentrations of FSH throughout the culture period, influenced the survival, growth and antrum formation of *in vitro* cultured goat preantral follicles. In experiment 1, preantral follicles were cultured in α-MEM$^+$ or α-MEM$^+$ supplemented with increasing concentrations of FSH throughout the culture period (sequential medium: FSH 100 ng/ml (days 0 to 6), FSH 500 ng/ml (days 6 to 12) and FSH 1000 ng/ml (days 12 to 18). The expression of luteinizing hormone receptor (LHR) was analyzed in noncultured and cultured follicles using real time RT-PCR. In experiment 2, isolated preantral follicles were cultured for 18 days in a sequential medium containing FSH (control) or a control medium supplemented with LH (50 or 100 ng/ml) from day 12 of culture onwards. Follicle development was evaluated on the basis of antral cavity formation as well as follicular and oocyte growth after *in vitro* maturation. FSH stimulated a significant increase in the expression of mRNA for LH receptors after 18 days of culture. Furthermore, after 18 days, all tested media promoted follicular survival and antrum formation; however, a significant increase in the rate of follicular growth and resumption of meiosis was observed when LH was used compared to the control. In conclusion, preantral follicles cultured in a medium supplemented with FSH increased LH receptor mRNA levels. Moreover, the addition of LH to the culture medium containing increasing concentrations of FSH (sequential medium) improved the *in vitro* development of goat preantral follicles.

Keywords: culture, FSH, goat, LH, LH receptor, preantral follicle.

Introduction

The domestic ruminant represents a valuable model system for the elucidation of endocrine and local mechanisms that control both the early and final stages of follicle development in monovulatory species. In humans, limited availability of suitable ovarian tissue is a major constraint to research in this area, and monovulatory domestic ruminants represent a physiologically relevant model to elucidate basic mechanisms before moving on to more focused clinical investigations (Campbell *et al*., 2003). However, to address common causes of infertility and to devise innovative strategies to increase the efficiency of assisted reproduction technologies, it is necessary to understand the basic physiology underlying the complex process of folliculogenesis.

Among the main substances that regulate the complex mechanisms of folliculogenesis are the pituitary gonadotropins FSH and LH, key hormones that regulate gametogenesis and steroidogenesis in the ovary. *In vivo*, preantral follicles are considered to be gonadotropin independent because animal and human preantral follicles can develop to the antral stage in conditions of minimal circulating gonadotropins (Gulyas *et al*., 1977; Halpin *et al*., 1986; Hillier, 1994; Cain *et al*., 1995). However, several *in vitro* studies have suggested that the development of early follicles occurs under the influence of gonadotropins (Cortvrindt *et al*., 1998a,b; Gutierrez *et al*., 2000; Wu *et al*., 2000; Itoh *et al*., 2002, Xu *et al*., 2009).

The cell surface receptors that mediate the biological activities of these hormones are thought to be expressed in a cell-specific fashion in the ovary and are regulated as individuals progress through the reproductive cycle (Camp *et al*., 1991). Expression of FSH receptor mRNA was recently demonstrated in primary, secondary and antral follicles in goat ovaries (Saraiva *et al*., 2010), while mRNA for LHR has been observed from secondary follicles onward in the bovine species (Braw-Tal and Roth, 2005). LH receptors first develop on the cells of the theca interna at the preantral stage of development, and this pattern of expression is maintained to the preovulatory stage (Richards *et al*., 1995). In addition, it is well established that the granulosa cells of large estrogenic antral follicles also develop LH receptors (Webb *et al*., 1999; Garverick *et al*., 2002).

In goats, FSH may be important for follicular development *in vitro* because its use is crucial for maintaining the survival and activation of early preantral follicles enclosed in ovarian tissue fragments

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(Matos *et al*., 2007, Magalhães *et al*., 2009). Moreover, in advanced preantral follicles, FSH stimulated antrum formation and growth, as well as meiotic resumption of oocytes (Saraiva *et al*., 2010). Regarding LH, most research investigating its role in follicle development has focused on the action of this gonadotropin in late-stage follicles during the peri ovulatory period, but its role in preantral follicle development has not been well characterized (Xu *et al*., 2009). *In vitro* studies have demonstrated that LH is a survival and differentiation factor that increases oocyte maturation in FSH-supplemented cultures of mouse (Cortvrindt *et al*., 1998a,b) and pig (Wu *et al*., 2007) preantral follicles, and LH has a dependent stage in mouse preantral follicle development *in vitro* (Wu *et al*., 2000). Few studies have shown that the effect of LH on the *in vitro* development of preantral follicles depends on the follicular category and concentration used (Cortvrindt *et al*., 1998a,b; Wu *et al*., 2000; Tamilmani *et al*., 2005; Saraiva *et al*., 2008). Furthermore, a recent study with goat preantral follicles revealed that the moment of addition of LH to a culture medium containing FSH and epidermal growth factor (EGF) influences the development of the oocytes (Silva *et al*., 2011). However, a very small number of these oocytes became competent to resume meiosis.

In this same species, the use of LH with FSH in the culture of primordial follicles enclosed in ovarian fragments is important for maintaining follicular ultrastructural integrity (Saraiva *et al*., 2008). However, it is not known whether FSH influences LH receptor mRNA expression or if the FSH/LH interaction influences the *in vitro* development of isolated goat secondary follicles. Thus, the aims of this study were: 1) to evaluate the effect of FSH on LH receptor mRNA expression after *in vitro* culture of goat preantral follicles and 2) to verify the effect of FSH alone, or in combination with LH, on the *in vitro* development of goat secondary follicles.

Materials and Methods

Chemicals

Recombinant bovine FSH was purchased from Nanocore (São Paulo, SP, Brazil). Unless stated otherwise, LH, culture medium and other chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Source of ovaries

The ovaries (n = 44) used in experiment 1 (n = 20) and experiment 2 (n = 24) were collected from locally slaughtered adult cross-breed goats (*Capra hircus*). Immediately following their collection, the ovaries were washed once with 70% alcohol for 10 sec and then twice with Minimum Essential Medium (MEM), which was buffered with HEPES (MEM-HEPES) and supplemented with penicillin (100 µg/ml) and streptomycin (100 µg/ml). The ovaries were maintained at 4°C and transported to the laboratory within 1 h of collection (Chaves *et al*., 2008).

Experiment 1: In vitro culture of isolated preantral follicles to analyze the steady-state level of LH receptor mRNA

Follicular isolation and culture

In the laboratory, surrounding fat tissue and ligaments were stripped from 10 pairs of ovaries. Ovarian cortical slices (1 to 2 mm in diameter) were cut from the ovarian surface using a surgical blade under sterile conditions. The ovarian cortex was subsequently placed in fragmentation medium consisting of MEM plus HEPES (pH 7.2 to 7.4), supplemented with 100 µg/ml penicillin and 100 µg/ml streptomycin. Preantral follicles ≥150 µm in diameter were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and manually dissected from strips of ovarian cortex using 26 gauge needles. After isolation, follicles were transferred to 100 µl drops of fresh medium and maintained under mineral oil to further evaluate follicular quality. Follicles with a visible oocyte that was surrounded by granulosa cells and had intact basement membrane and no antral cavity were selected for culture.

After selection, some follicles (n = 40) were submitted for analysis as the noncultured control, while others (n = 80 follicles) were individually cultured in 100 µl drops of culture medium under mineral oil in petri dishes (60 x 15 mm, Corning, USA). The basic culture medium consisted of α-MEM (pH 7.2 to 7.4) supplemented with 3.0 mg/ml bovine serum albumin (BSA), ITS (insulin 10 µg/ml, transferrin 5.5 µg/ml and selenium 5 ng/ml), 2 mM glutamine, 2 mM hypoxanthine and 50 µg/ml of ascorbic acid (called α-MEM$^+$). Incubation was conducted at 39°C and 5% CO_2 in air for 18 days. Fresh medium was prepared and incubated at 39°C for 1 h prior to use. For this experiment, 120 late preantral follicles were isolated and randomly distributed in the following treatments: fresh control (n = 40), α-MEM$^+$ or noncultured control (n = 40) and α-MEM$^+$ supplemented with increased concentrations of recombinant FSH throughout the culture period (n = 40). FSH concentrations were as follows: FSH 100 ng/ml (days 0 to 6), FSH 500 ng/ml (days 6 to 12) and FSH 1000 ng/ml (days 12 to 18). Every other day, 60 µl of the culture medium was replaced with fresh medium, except on days 6 and 12, when total medium replenishment was performed to ensure the appropriate concentration of FSH. After 18 days of culture, viable follicles were frozen at -80°C for further evaluation of LH receptor mRNA expression.

RT- qPCR analyses

Isolation of total RNA from noncultured and

cultured follicles (control and FSH) was performed using a Trizol plus purification kit (Invitrogen, São Paulo, Brazil). In accordance with the manufacturer's instructions, 1 ml of Trizol solution was added to each frozen sample and the lysate was aspirated through a 20 gauge needle before centrifugation at 10,000 g for 3 min at room temperature. Thereafter, all lysates were diluted 1:1 with 70% ethanol and subjected to a mini-column. After binding of the RNA to the column, DNA digestion was performed using RNase-free DNase (340 Kunitz units/ml) for 15 min at room temperature. After washing the column three times, the RNA was eluted with 30 µl RNase-free water.

Prior to reverse transcription, the eluted RNA samples from follicles from all treatments were incubated for 5 min at 70°C and chilled on ice. Reverse transcription was then performed in a total volume of 20 µl, which was comprised of 10 µl of sample RNA, 4 µl 5X reverse transcriptase buffer (Invitrogen), 8 units RNAseOut, 150 units Superscript III reverse transcriptase, 0.036 units random primers (Invitrogen), 10 mM DTT, and 0.5 mM of each dNTP. The mixture was incubated for 1 h at 42°C, for 5 min at 80°C and then stored at -20°C. From this reverse transcription reaction, complementary DNAs (cDNAs) were obtained. Negative controls were prepared under the same conditions but without the inclusion of the reverse transcriptase.

PCR reactions were composed of 1 µl cDNA in 7.5 µl of SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA, USA), 5.5 µl of ultra-pure water and 0.5 µm of each primer. The primers were designed to perform amplification of LH receptor mRNA and β-actin and Ubiquitin (Table 1) were used as endogenous controls for normalization of steady-state levels of mRNA of the LH gene. The thermal cycling profile for the first round of PCR was as follows: initial denaturation and activation of the polymerase for 15 min at 94°C followed by 40 cycles of 15 sec at 94°C, 30 sec at 60°C and 45 sec at 72°C. The final extension was for 10 min at 72°C. All reactions were performed in a real-time PCR Mastercycler (Eppendorf, Germany). The delta-delta-CT method was used to transform CT values into normalized relative steady-state levels of mRNA.

Table 1. Oligonucleotide primers used for the analysis of goat follicles by polymerase chain reaction (s = sense, as = antisense).

Target gene	Primer sequence (5`→3`)	Sense	Position	GenBank accession number
β-actin	ACCACTGGCATTGTCATGGACTCT	s	187-211	GI: 28628620
	TCCTTGATGTCACGGACGATTTCC	as	386-410	
Ubiquitin	GAAGATGGCCGCACTCTTCTGAT	s	607-631	GI: 57163956
	ATCCTGGATCTTGGCCTTCACGTT	as	756-780	
LHR	CGATTTCACCTGCATGGCACCAAT	s	14-37	GI: 20977242
	GATTGGCGCATGAATTGACGGGAT	as	141-164	

Experiment 2: In vitro culture of isolated preantral follicles to maturation

For this experiment, preantral follicles were isolated from 12 ovarian pairs and cultured as described in experiment 1. Approximately 40 follicles were used per treatment for a total of 124 follicles. Isolated follicles were randomly distributed in the following treatments: α-MEM$^+$ supplemented with increased concentrations of recombinant FSH throughout the culture period (FSH 100 ng/ml from day 0 to day 6, FSH 500 ng/ml from day 6 to day 12 and FSH 1000 ng/ml from day 12 to day 18), corresponding to the control medium (control group).The control medium was also supplemented with 50 ng/ml LH (LH 50 ng/ml group) or 100 ng/ml LH (LH 100 ng/ml group) from day 12 of culture onward. Every other day, 60 µl of the culture medium were replaced with fresh medium, except on days 6 and 12, when total medium replenishment was performed to ensure the appropriate concentration of FSH.

Follicles were classified according to morphological characteristics, and those showing morphological signs of degeneration, such as darkness of oocytes and surrounding cumulus cells or those with misshapen oocytes, were classified as degenerated. Oocyte and follicular diameter were measured only in healthy follicles at x and y dimensions (90°) using an ocular micrometer (100X magnification) inserted into a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) on day 0 and day 18 of culture. The follicle diameter growth rate was calculated as follows: the diameter of viable follicles at day 18 minus the diameter of viable follicles at day 0 divided by the number of days of *in vitro* culture (18 days). Antral cavity formation was defined as a visible translucent cavity within the granulosa cell layers.

In vitro maturation of oocytes from cultured preantral follicles

At the end of the 18-day culture period, all healthy follicles were carefully and mechanically opened with 26 gauge needles under a stereomicroscope for oocyte recovery. Only oocytes ≥110 µm with homogeneous cytoplasm and surrounded by at least one

compact layer of cumulus cells were selected for *in vitro* maturation (IVM). The oocyte recovery rate (ORR) was calculated by dividing the number of recovered oocytes for IVM by the total number of follicles on day 18 of culture and multiplying the result by 100. The selected COCs were washed three times in a maturation medium composed of TCM-199 supplemented with 1 mg/ml bovine serum albumin (BSA), 5 µg/ml pituitary LH, 0.5 µg/ml rFSH, 1 µg/ml 17β-estradiol, 10 ng/ml epidermal growth factor (EGF), 50 µg/ml insulin-like growth factor (IGF-1), 100 µM cysteamine and 1 mM pyruvate. Using this medium in our laboratory, the mean maturation rate of COCs recovered from antral follicles was 80% (Figueiredo *et al.*, State University of Ceará, Fortaleza, CE, Brazil, 2011; unpublished data). After washing, the oocytes were transferred to 50 µl drops of maturation medium under mineral oil and incubated for 32 h at 39ºC with 5% CO_2 in air.

At the end of the maturation period, COCs were analyzed under fluorescent microscopy for assessment of viability and chromatin configuration. The COCs were quickly incubated in 100 µl droplets of TCM-199 containing 4 µM calcein-AM, 2 µM ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany) and 10 µM Hoechst 33342 (Bisbenzimide trihydrochloride) at 37°C for 15 min. Afterwards, the COCs were washed three times in TCM-199 and examined under a fluorescent microscope (Nikon, Eclipse 80i, Tokyo, Japan). The emitted fluorescent signals of calcein-AM, ethidium homodimer-1 and Hoechst were collected at 488, 568 and 365 nm, respectively. Oocytes and granulosa cells were considered live if the cytoplasm was stained positively with calcein-AM (green) and if the chromatin was not labeled with ethidium homodimer-1 (red). In addition, the chromatin configuration was analyzed when the oocytes were labeled with Hoechst 33342 (blue).

Statistical analyses

Data referring to LH receptor mRNA expression after culture were analyzed by paired Student's *t*-test ($P < 0.05$). Data concerning follicular survival, antrum formation and meiotic resumption after *in vitro* culture were analyzed as dispersion of frequency, being expressed as percentages and compared by Chi-square test. Data from follicle and oocyte diameters were initially submitted to Shapiro-Wilk and Bartlett's tests to confirm normal distribution and homogeneity of variance, respectively. An analysis of variance was then carried out using the PROC GLM procedure of SAS, 1999. For both experiments (1 and 2), four replicates were performed for each treatment. Isolated follicles were considered as the experimental unit, and the following general model was used:

$$Y_{ijk}=\mu+M_i+P_j+T_k+(M_i\acute{}P_j)+(M_i\acute{}T_k)+(P_j\acute{}T_k)+(M_i\acute{}P_j\acute{}T_k)+e_{ijk}$$

where Y_{ijk} = dependent variable (follicle diameter), μ = general mean, M_i = presence or not of FSH in the medium, P_j = LH concentrations, T_k = time culture, $M_i\acute{}P_j$ = interaction between medium and LH concentration, $M_i\acute{}T_k$ = interaction between medium and culture time, $P_j\acute{}T_k$ = interaction between LH concentration and culture time, $M_i\acute{}P_j\acute{}T_k$= interaction among medium, LH concentration and culture time and e_{ijk} = residual error. For growth rate, the model included only medium and LH concentrations because data were calculated at the end of culture time. When any main effect or interactions were significant, means were separated by least significant difference. Differences were considered to be significant when $P < 0.05$, and results were expressed as percentage or mean ± standard deviation (SD).

Results

Experiment 1

Following analysis of the levels of LH receptor mRNA after 18 days of culture (Fig. 1), it was observed that the level of expression was higher ($P < 0.05$) in follicles cultured with FSH compared to fresh control (noncultured - day 0) and those cultured in α-MEM$^+$ alone (cultured control).

Experiment 2

Table 2 shows the percentages of follicle survival and antrum formation, daily follicular growth rate and the recovery rate of fully grown oocytes (≥110 µm) after *in vitro* culture of goat preantral follicles at different concentrations of LH (50 and 100 ng/ml). Figure 2 illustrates preantral follicles before culture (Fig. 2A and 2B) and after antrum formation on days 6, 12 and 18 of culture (Fig. 2C, 2D and 2E, respectively). At the end of culture, there was no significant difference among treatments in relation to follicular survival, antrum formation and recovery rate of the oocyte. Nevertheless, the addition of LH in both concentrations (50 and 100 ng/ml) induced a significant increase in daily follicular growth rate when compared to the control group ($P < 0.05$).

After 18 days of culture, oocytes from healthy follicles that had a minimum diameter of 110 µm were recovered and destined for IVM. As shown in Table 3, the addition of 100 ng/ml LH resulted in a significantly higher percentage of oocytes at metaphase I compared to control ($P < 0.05$), but it was similar to the addition of 50 ng/ml LH. Oocytes at different stages of maturation can be seen in Fig. 3.

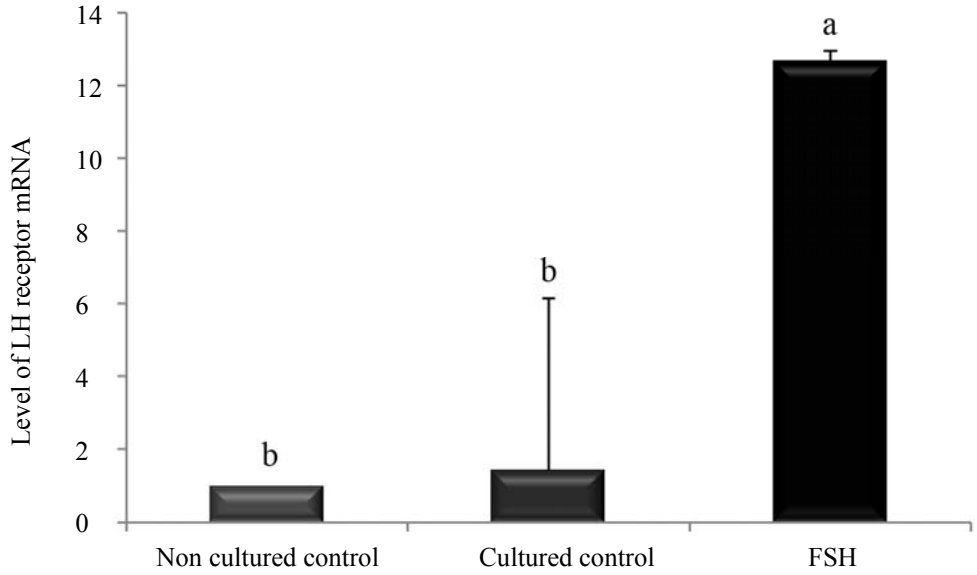

Figure 1. Steady-state level of LH receptor mRNA in goat secondary follicles non-cultured (day 0) or cultured for 18 days in α-MEM$^+$ or α-MEM$^+$ with FSH. ^{a,b}Differs among treatments ($P < 0.05$; experiment 1).

Table 2. Percentages of survival and antrum formation and daily follicular growth rate of oocytes destined to IVM after 18 days of culture in preantral follicles in control medium or with different concentrations of LH (experiment 2).

Treatments	Follicles cultured	Survival follicles (%)	Antrum formation (%)	Growth/day (µm)	RRO (%)
Control	42	36 (85.71)^A	37 (88.1)^A	17.3 ± 7.9^B	33 (78.6)^A
LH 50 ng/ml	42	29 (69.05)^A	41 (97.6)^A	23.7 ± 6.2^A	33 (78.6)^A
LH 100 ng/ml	40	29 (72.50)^A	36 (90.0)^A	22.6 ± 9.0^A	32 (80.0)^A

^{A,B}Differs among treatments in the same column ($P < 0.05$). RRO: Recovery rate of oocyte ≥110 µm.

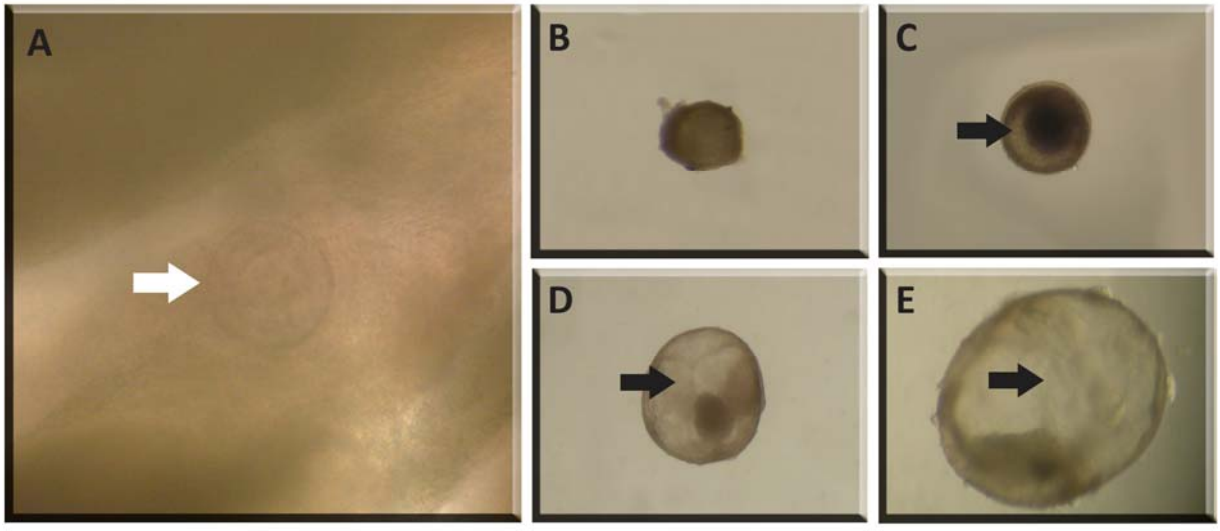

Figure 2. Goat follicles before and after culture. A) Goat preantral follicle enclosed in ovarian tissue (white arrow); (B) Goat preantral follicle isolated on day 0 of culture; (C) Goat preantral follicle from the control group after 6 days of culture showing early antrum cavity (black arrow); (D) Goat antral follicle from the FSH+LH 100 ng/ml group after 12 days of culture showing the antrum cavity (black arrow); (E) Goat antral follicle from the FSH+LH 100 ng/ml group after 18 days of culture showing a large antrum cavity (black arrow).

Table 3. Percentages of oocytes showing germinal vesicle (GV), germinal vesicle breakdown (GVBD) and in metaphase I (MI) after IVM (experiment 2).

Treatments	Viable oocytes ≥110 µm	GV (%)	GVBD + MI (%)	MI (%)
Control	30	14 (46.7)^{A}	13 (43.3)^{A}	0 (0.00)^{B}
LH 50 ng/ml	33	14 (42.4)^{A}	14 (42.4)^{A}	3 (9.1)^{AB}
LH 100 ng/ml	29	11 (40.0)^{A}	15 (51.7)^{A}	7 (24.1)^{A}

^{A,B}Differs among treatments in the same column ($P < 0.05$).

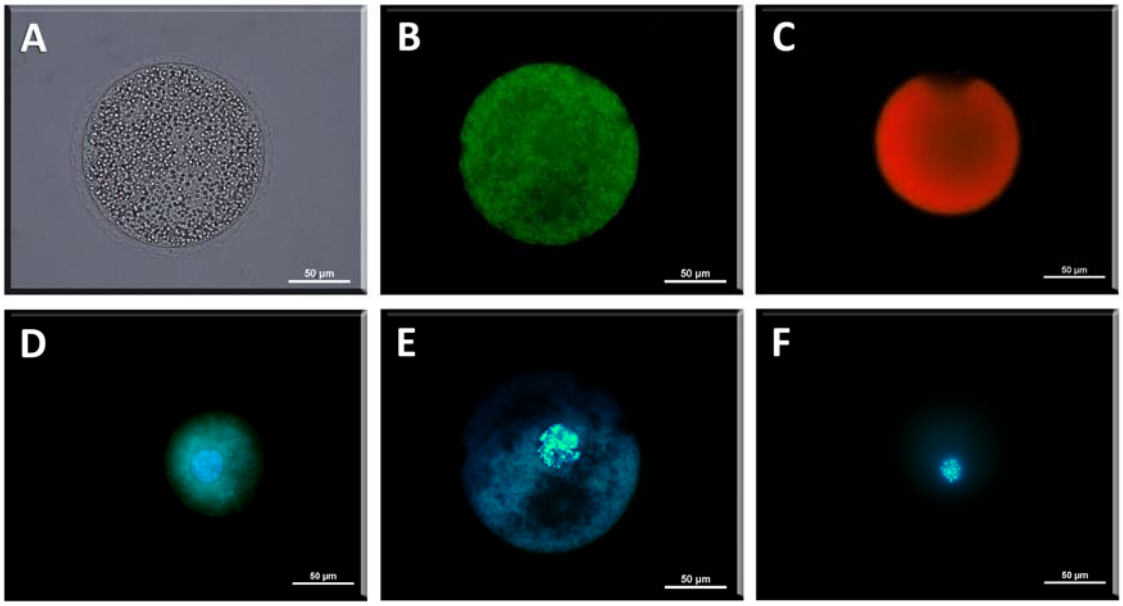

Figure 3. Goat oocytes after *in vitro* growth and maturation of preantral follicles. (A) Non-stained oocyte (diameter ≥110 µm) mechanically denuded after maturation; (B) Viable oocyte from the FSH+LH 50 ng/ml group labeled with calcein-AM; (C) Non-viable oocyte from the control group labeled with ethidium homodimer-1; (D) Oocyte from the control group showing an intact nuclear membrane (germinal vesicle stage); (E) Oocyte from the FSH+LH 50 ng/ml group showing germinal vesicle breakdown; (F) Oocyte from the FSH+LH 100 ng/ml group showing metaphase I labeled with Hoechst 33342.

Discussion

This study demonstrates that FSH increases the expression of LH receptor mRNA levels during culture and that the association between the gonadotropins (FSH and LH) influences the *in vitro* development of secondary follicles in goats. A previous study performed by our team, involving the *in vitro* culture of goat primordial follicles enclosed in ovarian tissue, showed that the association of FSH and LH was very important for the maintenance of follicular ultrastructure but had little effect on follicular growth (Saraiva *et al*., 2008). However, to our knowledge, no information is available concerning the effect of these gonadotropins on the *in vitro* development of late secondary follicles in goats. Given this lack of information, there was a need to further investigate the function of LH and its interaction with FSH on the development of advanced preantral follicles. Therefore, in the present study we used a sequential basic medium (developed in our laboratory) which established that the use of defined concentrations of FSH, added at specific times during culture in a progressive way, is essential for the *in vitro* development of goat preantral follicles (Saraiva *et al*., 2010).

Using this sequential medium, we observed in the present study that FSH stimulates an increase in the expression of LH receptor mRNA after 18 days of culture. LH receptor mRNA is located predominantly in theca cells of small follicles and then appears in the granulosa cells of growing follicles (Camp *et al*., 1991). Previous studies have confirmed that FSH induces the appearance of LH receptors on granulosa cells of ovarian follicles both *in vivo* and *in vitro* (Zeleznik *et al*., 1974; Nimrod *et al*., 1977; Nimrod, 1981). In addition, similar to our *in vitro* results, it was observed that LH receptors increase with the progression of follicle growth *in vivo* (Braw-Tal and Roth, 2005) and that the expression of these receptors changes considerably during the ovarian cycle (LaPolt *et al*., 1990; Segaloff *et al*., 1990; Hoffman *et al*., 1991). Increased expression of LH receptor mRNA during the culture of secondary follicles in medium containing sequential FSH suggests that the addition of LH to the

culture medium after FSH stimulation is important for follicular development at this stage. It has been reported that the effect of LH on the *in vitro* culture of preantral follicles depends on the follicular category, the concentration used and the timing of the addition of LH to the culture medium (Cortvrindt *et al*., 1998a, b; Wu *et al*., 2000; Tamilmani *et al*., 2005; Saraiva *et al*., 2008; Silva *et al*., 2011). The addition of LH or LH plus FSH to the culture medium starting on day 0 was detrimental to *in vitro* survival and follicular development (Tamilmani *et al*., 2005, Xu *et al*., 2009) and for this reason, in this study LH was added only after the day 12, when almost all the follicles in all treatments exhibited antrum formation. Furthermore, studies have shown that exposure to LH prior to reaching an appropriate stage of development may disrupt signaling in the follicle, thereby preventing proper follicle maturation (Xu *et al*., 2009).

In the present study, the culture system that was employed allowed for the maintenance of follicular survival, antrum formation and continuous follicular growth in all treatments. However, it was observed that the association of FSH and LH at both concentrations (50 and 100 ng/ml) was more effective in promoting follicular growth compared to FSH alone. This observation may be related to the fact that FSH increased the expression of LH receptors after 18 days of culture, promoting a synergistic effect of both gonadotropins on *in vitro* follicular development. Moreover, it is known that although the action of LH is targeted for the final stages of folliculogenesis, its joint action with FSH contributes to cell proliferation and differentiation, estrogen production and subsequent maturation of oocytes from cultured preantral follicles (Qvist *et al*., 1990). Similar to our findings, FSH/LH interaction has also been successfully demonstrated in mice, where *in vitro* culture of preantral follicles with a diameter of 100-130 µm using both gonadotropins (LH 10 mIU/ml and FSH 100 mIU/ml) in the presence of fetal calf serum (5%) stimulated antrum formation more efficiently than FSH alone after 12 days of culture (Ola *et al*., 2008). It has been suggested that low LH supplementation during primary and secondary follicle culture enables follicles to respond to later LH-dependent growth (Wu *et al*., 2000). In previous reports of follicle culture, LH supplementation did not affect follicle survival, but did enhance follicle growth and antrum formation in the mouse and human, increasing the rate of oocyte maturation to the metaphase II stage in mice (Abir *et al*., 1997; Cortvrindt *et al*., 1998b). On the other hand, studies with nonhuman primates (*Rhesus monkeys*) showed that the addition of LH during culture with FSH promoted a negative effect on survival and follicular growth and the best results were obtained when FSH was used alone (Xu *et al*., 2009).

In this study, high rates of meiosis resumption (GVBD+MI) were observed in all evaluated groups (42%). Our results were superior to those verified by Silva *et al*. (2011) after *in vitro* culture of goat preantral follicles using different moments of addition of LH to medium containing sequential FSH and EGF (~22% meiosis resumption). Based on this finding, we hypothesize that the beneficial effect of the use of sequential FSH alone or in combination with LH on induction of meiotic competence is impaired by the addition of EGF to the culture medium.

In the present work, it was also observed that the interaction of 100 ng/ml LH with FSH increased the percentage of oocytes at metaphase I compared to FSH alone. Similarly, Gao *et al*. (2007) observed that LH caused a spontaneous resumption of meiosis, whereas FSH alone rarely promoted germinal vesicle breakdown of oocytes from mouse preantral follicles cultured *in vitro*. In this same species, more significant results were obtained when both gonadotropins (LH 10 or 100 mIU/ml and FSH 100 mIU/ml) were used in the culture of preantral follicles 100-120 µm in diameter, resulting in the maturation of preantral follicles, acquisition of meiotic competence of mature oocytes, fertilization and subsequent blastocyst formation (Liu *et al*., 2002). Cortvrindt *et al*. (1998b) found that LH generated favorable conditions for the transition of oocytes from metaphase I to metaphase II in mice. In pigs, the addition of LH associated with FSH stimulated growth and improved the quality of oocytes compared to the control after 3 days of *in vitro* culture of preantral follicles (Wu *et al*., 2000).

In conclusion, FSH increases the levels of LH receptor mRNA during *in vitro* culture of secondary follicles, ensuring a synergistic effect of both gonadotropins on follicular growth and meiotic resumption of oocytes recovered from goat preantral follicles cultured *in vitro*.

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