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# Pituitary porcine FSH, and recombinant bovine and human FSH differentially affect growth and relative abundances of mRNA transcripts of preantral and early developing antral follicles in goats

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ABSTRACT

Three different sources of FSH (porcine pituitary, pFSH; recombinant bovine, rbFSH; and recombinant human, rhFSH) were compared during *in vitro* culture of preantral and early antral follicles of goats for 18 days. Treatments were: base medium supplemented with no FSH (control), 10, 50, or 100 mIU/mL pFSH (pFSH10, pFSH50, and pFSH100, respectively), 100 ng/mL rbFSH (rbFSH), and 50 mIU/mL rhFSH (rhFSH). There were evaluations of follicle morphology, antrum formation, growth rate, estradiol production, oocyte viability and chromatin configuration, and follicle wall relative abundance of mRNA transcript for *MMP-9*, *TIMP-2*, *CYP17*, *CYP19A1, FSHR, Insulin-R*, and *BAX/BCL-2 ratio*. Follicle degeneration rates were similar among all treatment groups at the end of culturing. When there were treatments with pFSH, however, there was a lesser ($P < 0.05$) percentage of intact follicles and estradiol production, and greater ($P < 0.05$) extrusion rates. Furthermore, with only pFSH10 (antral follicles) and pFSH100 (preantral and antral follicles) treatments, there was a lesser ($P < 0.05$) follicle growth. For preantral follicles, when there was addition of pFSH10, pFSH100, and rhFSH there was lesser ($P < 0.05$) oocyte meiotic resumption compared to control and rbFSH treatments. For antral follicles, when there were treatments with rhFSH and pFSH10 there was greater ($P = 0.08 - P < 0.05$) oocyte maturation. In conclusion, the source of FSH differentially affected gene expression, as indicated by mRNA abundances, and follicular dynamics of preantral and antral follicles *in vitro*. Addition of FSH during the *in vitro* culture improved the developmental outcomes only for antral follicles.

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## 1. Introduction

The ovarian follicular population is composed mainly of preantral follicles (90–95%), which constitute the major oocyte source for utilization of reproductive technologies. The majority of these follicles undergo atresia during the growth and development stages. Follicle recovery followed by *in vitro* follicle culture may increase oocyte availability for gaining a greater understanding of the mechanisms involved in ovarian folliculogenesis and complement other technologies such as *in vitro* embryo production (Arunakumari et al., 2010).

For several years, different culture systems have been developed (Van Den Hurk et al., 2000) to determine the appropriate culture conditions to support follicular development *in vitro* and to produce competent *in vitro*-developed oocytes. Ovarian preantral follicles in farm animals are usually cultured enclosed in ovarian tissue (*in situ*) or in an isolated state. The *in situ* culture allows for studying early folliculogenesis by evaluating substances that might have an effect on follicular activation and early development (Fortune, 2003). Furthermore, it maintains the interaction between the follicle and surrounding stromal cells (Goncalves et al., 2008). The *in vitro* culture of isolated follicles, however, allows for individual follicle assessment during periods of culturing and enhances the capacity for perfusion of culture medium into the follicular milieu (Abir et al., 2006). This system is commonly used for the *in vitro* culture of secondary follicles (more advanced preantral follicle stage) in several farm animals, such as goats (Thomas et al., 2003).

Among the hormones that may potentially affect the survival and development of preantral follicles *in vitro* is the follicle-stimulating hormone (FSH). This hormone is a pituitary-produced gonadotropin that affects follicular growth and development (Brown and McNeilly, 1999; Richards et al., 2002). The FSH receptor (FSHR) mRNA transcript was detected in granulosa cells of preantral and antral follicles of cattle (Xu et al., 1995), as well as in granulosa cells of goat small antral follicles (Saraiva et al., 2011). Furthermore, there has been detection of FSHR protein in granulosa cells of pig and human primary follicles onwards (Méduri et al., 2002), and secondary and antral follicles of goats (Barros et al., 2013). Follicle development, survivability, and proliferation of granulosa cells of preantral follicles in mice (Adriaens et al., 2004), women (Wright et al., 1999), sheep (Mbemya et al., 2018), and goats (Saraiva et al., 2010) have been reported after FSH use in culture. Furthermore, this hormone seems to have an important function in follicular atresia by preventing apoptosis in preantral and antral follicles (Wright et al., 1999). Different sources of FSH have been used for the *in vitro* culture of follicles in several species: porcine pituitary FSH (pFSH) (caprine: Magalhães et al., 2009b), recombinant human FSH (rhFSH) (murine: Adriaens et al., 2004; caprine: Rocha et al., 2014; ovine: Barros et al., 2019; non-human primate: Xu et al., 2010), and recombinant bovine FSH (rbFSH) (caprine: Saraiva et al., 2011; Ferreira et al., 2016; equine: Aguiar et al., 2016; canine: Serafim et al., 2013). Studies in which there was comparison of different sources of FSH in the same experimental conditions are scarce. In addition, the concentrations of FSH used have been extremely important when evaluating effects of this hormone (e.g., 1 to 2000 mIU/mL: Naydu and Osborn, 1992; Ihm et al., 2015; and 50 to 1000 ng/mL: Saraiva et al., 2011). For example, Magalhães et al. (2009a) compared pFSH and rbFSH during the *in vitro* culture of preantral follicles in ovarian tissue fragments (*in situ*) and reported that rbFSH was a more desirable option than pFSH regarding follicle stimulation and induction of morphological changes.

The *in vitro* culture system for ovarian follicles has been improved during the last decades for animal models (O'Brien, 2003; Shankaraiah et al., 2018), and humans (McLaughlin et al., 2018). In goats, the most encouraging results for *in vitro* follicle culture was the development of a morula from oocytes obtained from secondary follicles cultured in the isolated form (Magalhães et al., 2011). Furthermore, results from recent studies indicate there is a stage-specific response of ovarian follicles to similar culture conditions with lesser oocyte maturation rates (Metaphase II (MII) oocytes) in preantral follicles compared to early-stage antral follicles (Cadenas et al., 2017; Ferreira et al., 2018).

The objective and originality of the present study was to compare the *in vitro* culture of isolated preantral and early antral follicles using three different sources and concentrations of FSH (recombinant bovine, recombinant human, and porcine pituitary) when there were the same experimental conditions (e.g., ovary pair, incubator, and operator). The following end points were evaluated: (1) follicle growth and morphology; (2) oocyte diameter, viability, and maturation; (3) estradiol production; and (4) abundance of *MMP-9*, *TIMP-2*, *CYP17*, *CYP19A1*, *FSHR*, *Insulin-R* mRNA transcripts and *BAX/BCL-2 ratio* mRNA transcript ratio.

## 2. Materials and methods

### 2.1. Chemicals and media

The reagents and chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

### 2.2. Ovaries, and preantral and early antral follicle manipulation

The ovaries ($n = 90$) from sexually mature crossbred goats (ages 1–3 years) were collected at a slaughterhouse and transported to the laboratory in MEM-HEPES within 1 h at 4°C, as described previously (Chaves et al., 2008). Preantral (approximately, 200–300 μm in diameter) and early antral morphologically intact follicles (approximately, 300–400 μm in diameter) were manually dissected and selected as previously described (Cadenas et al., 2017). Selected follicles were individually cultured in 100 μL drops of culture medium in Petri dishes. The base culture medium (control treatment) consisted of α-MEM (pH 7.2–7.4, Gibco; Invitrogen, Karlsruhe, Germany) supplemented with 3 mg/mL bovine serum albumin (BSA), 5.5 μg/mL transferrin, 5 ng/mL selenium, 2 mM glutamine, 2 mM hypoxanthine, 50 μg/mL ascorbic acid, and 10 μg/mL human recombinant insulin, and was referred to as α-MEM$^+$ (Ferreira

et al., 2016). The culture period lasted 18 days and was performed at 39°C in 5% $CO_2$ in air. Fresh medium was prepared 2 h before use, and 60 μL of medium was refreshed in each drop every 2 days. Approximately 40 follicles were used per treatment and five replicates were performed.

### 2.3. Experimental design

Preantral and early antral follicles were randomly assigned to the following treatment groups: α-MEM$^+$ alone (control); α-MEM$^+$ supplemented with porcine pituitary FSH (pFSH) at doses of 10 mIU/mL (pFSH10), 50 mIU/mL (pFSH50), and 100 mIU/mL (pFSH100); α-MEM$^+$ supplemented with recombinant human FSH (rhFSH) at 50 mIU/mL (~3.7 ng/mL); and α-MEM$^+$ supplemented with recombinant bovine FSH (rbFSH) at 100 ng/mL. The recombinant bovine FSH (100 ng/mL) and recombinant human FSH (50 mIU/mL) concentrations were selected based on previous dose-response studies (Ferreira et al., 2016, 2018, respectively).

### 2.4. In vitro follicle growth and oocyte maturation

Follicles were morphologically classified every 6 days of culture, as intact, extruded, or degenerated; and the diameter of morphologically intact follicles were recorded on days 0, 6, 12, and 18 of culture and the follicular growth rate was calculated as previously described (Ferreira et al., 2018). The oocyte recovery rate was calculated by dividing the number of oocytes ≥110 μm in diameter (zona pellucida not included) by the number of viable follicles at the end of the culture period and multiplying this value by 100. After the follicle culture periods were completed, there was an incision made in all morphologically normal follicles and oocytes with homogeneous cytoplasm surrounded by at least one compact layer of cumulus cells were selected for *in vitro* maturation (IVM). The IVM medium was composed of tissue culture medium 199 (TCM 199) supplemented with 1 μg/mL 17β-estradiol, 5 μg/mL luteinizing hormone (LH), 0.5 μg/mL rFSH, 10 ng/mL epidermal growth factor (EGF), 1 mg/mL BSA, 1 mM pyruvate, 50 ng/mL insulin-like growth factor 1 (IGF-I), and 100 μM cysteamine. The selected cumulus-oocyte complexes (COC) were rinsed three times in IVM medium and individually transferred to fresh IVM medium (1 COC/10 μL droplet) on culture dishes under mineral oil and incubated for 32 h at 39°C with 5% $CO_2$ in air (Cadenas et al., 2018).

### 2.5. Assessment of oocyte viability and chromatin configuration

The evaluation of oocyte viability and maturation (i.e., chromatin configuration) was conducted using fluorescence microscopy (Nikon, Eclipse 80i, Tokyo, Japan). After IVM, COC were mechanically denuded and incubated individually in 10 μL drops of PBS supplemented with 2 μM ethidium homodimer-1, 4 μM Calcein-AM (Molecular Probes – LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells – L3224; Invitrogen, Karlsruhe, Germany), 5.5 μg/mL Hoechst 33342, and 1% glutaraldehyde at room temperature for 30 min, and processed as described previously (Cadenas et al., 2017). Oocyte chromatin was classified as a germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), or metaphase II (MII). Meiotic resumption was considered to have occurred when there was the presence of GVBD, MI, and MII oocytes.

### 2.6. Follicular wall RNA extraction and real-time PCR (qPCR)

After culture, three pools of ten follicular walls (containing granulosa and theca cells of preantral and early antral follicles) were collected from each treatment group for RNA isolation. Total RNA was isolated with Trizol® reagent method (Invitrogen, Carlsbad, CA, USA), and purified with PureLink™ RNA Mini Kit (Ambion®, Carlsbad, CA, USA). cDNA synthesis from 1 ng of total RNA was performed following the instructions of the Superscript III RT-PCR manual (Invitrogen, Carlsbad, CA, USA) using random primers (Invitrogen, Carlsbad, CA, USA). The primers were designed to perform the amplification of matrix metallopeptidase 9 (MMP9), tissue inhibitor of metalloproteinases 2 (TIMP2), cytochrome P450 17α-hydroxylase/17,20-lyase (*CYP17*), aromatase (*CYP19A1*), FSH receptor (*FSHR*), insulin receptor (*Insulin-R*), and *BAX/BCL-2* ratio mRNA abundances (Table 1). Two candidate reference genes, glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) and peptidylprolyl isomerase A (*PPIA*), were selected as endogenous controls to study the abundance and mRNA transcript stability, and for normalization of relative abundances of mRNA in all samples. All reactions were performed using an IQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), and the delta-delta-CT method (Livak and Schmittgen, 2001) was used to transform threshold cycle values (Ct) into normalized relative expression levels of mRNA (Silva et al., 2011).

### 2.7. Estradiol assay

The spent medium was individually collected on day 18 of culture and stored at −80°C until assay. Estradiol production was determined using an enzyme-linked fluorescent assay (ELFA) utilizing a commercial kit (Vidas® Estradiol II – 30431) and an automatic analyzer miniVIDAS® (bioMérieux SA, Lyon, France) (Ferreira et al., 2018). The intra-assay coefficient of variation and sensitivity of the assay were 5% and 9 pg/mL, respectively.

**Table 1**
Oligonucleotide primers used for PCR analysis of goat follicles.

| Target gene | Primer sequence (5'→3') | Sense/anti-sense * | GenBank accession nº |
|---|---|---|---|
| *FSHR* | AGGCAAATGTGTTCTCCAACCTGC | S | NM_001285636.1 (*Capra hircus*) |
| | TGGAAGGCATCAGGGTCGATGTAT | AS | |
| *Insulin-R* | ATGCCCTGGTGTCACTTTCCTTCT | S | XM_012177947.3 (*Ovis aries*) |
| | TTAGGTTCTGGTTGTCCAAGGCGT | AS | |
| *CYP19A1* | CGGCATGCATGAGAAAGGCATCAT | S | NM_001285747.1 (*Capra hircus*) |
| | ACACGTCCACATAGCCCAAGTCAT | AS | |
| *CYP17* | ACTGAATGCCTTTGCCCTGT | S | NM_001314145.1 (*Capra hircus*) |
| | CTGATTATGTTGGTGATCC | AS | |
| *MMP9* | TTTCCTCCTGGCTCAGGCATTCA | S | NM_001314269.1 (*Capra hircus*) |
| | GTTTCCGAAGTAGGTCGGGATCACA | AS | |
| *TIMP2* | AGAAGAAGAGCCTGAACCACAGGT | S | XM_018063674.1 (*Capra hircus*) |
| | TGATGTTCTTCTCCGTGACCCAGT | AS | |
| *BCL2* | ATG ACT TCT CTC GGC GCT AC | S | XM_027526258.1 (*Capra hircus*) |
| | ACG CTC TCC ACA CAC ATG AC | AS | |
| *BAX* | CTC TCC CCG AGA GGT CTT TT | S | XM_015458140.2 (*Capra hircus*) |
| | TGC AAG GAA GTC CAA TGT CC | AS | |
| *GAPDH* | ATGCCTCCTGCACCACCA | S | XM_027541122.1 (*Ovis aries*) |
| | AGTCCCTCCACGATGCCAA | AS | |
| *PPIA* | TCATTTGCACTGCCAAGACTG | S | XM_018047035.1 (*Capra hircus*) |
| | TCATGCCCTCTTTCACTTTGC | AS | |

*S, Sense; AS, Anti-sense.

### 2.8. Statistical analyses

All statistical analyses were performed using Sigma Plot version 11 (Systat Software Inc., USA). The proportion of follicular (morphologically normal, extruded, and degenerated follicles, antrum formation) and oocyte variables (viability and chromatin configuration) among treatments and days of culture were evaluated using the chi-square or Fisher's exact test. Comparison of means (follicle and oocyte diameter, follicular growth rate, and estradiol) were evaluated using the Kruskal-Wallis test and Wilcoxon Mann-Whitney test when appropriate, and potential differences in relative abundances of mRNA transcript were analyzed using t-tests. Data were evaluated for extreme values utilizing the Dixon outlier test (Zar, 1984), and outliers were excluded from any statistical analysis. Logistic regression analysis was used to evaluate the relationship (odds ratio, OR) of follicle morphology, antrum formation, and oocyte diameter (independent variables) with oocyte viability, meiotic resumption, and metaphase II rates (dependent variables). Differences were considered significant at $P < 0.05$ (two-sided). Probability values $> 0.05$ and $< 0.1$ indicate that a difference approached significance. Data are presented as a percentage or mean ( ± SEM).

## 3. Results

### 3.1. Morphology and growth of preantral and early antral follicles

At the onset of culture (day 0), a total of 263 preantral and 263 early antral morphologically intact follicles were randomly distributed among the different treatment groups and cultured *in vitro* for 18 days. Overall, there was a decrease ($P < 0.05$) in the percentage of morphologically intact follicles, while there was an increase ($P < 0.05$) in both follicle extrusion (i.e., rupture of the basement membrane with partial release of the follicle content) and degeneration throughout the culture period (Fig. 1). When comparing all treatments at the end of the culture period, with use of pFSH there were fewer ($P < 0.05$) morphologically intact follicles and more ($P < 0.05$) extruded follicles in both follicle categories. Furthermore, antrum formation was less ($P < 0.05$) when there was culturing with pFSH and rhFSH compared with the follicles in the control group (Fig. 2). Nevertheless, when there was addition of rbFSH to culture media, there was a similar ($P > 0.05$) rate of antrum formation compared with the follicles in the control group, but there were greater ($P < 0.05$) antrum formation rates with the rbFSH than with the pFSH100 treatment.

Follicle diameter increased ($P < 0.05$) during the culture period in all treatment groups compared to that on day 0 (Table 2). Nonetheless, only in the control, rbFSH, and rhFSH treatment groups did follicle diameter progressively increase ($P < 0.05$) every 6 days in both antral and preantral follicles, while with the pFSH100 treatment this only occurred in antral follicles. Preantral follicles cultured with 10, 50, or 100 mIU/mL pFSH were smaller ($P < 0.05$) in diameter at the end of the culture period than preantral follicles of the control group. For antral follicles, however, there was a smaller ($P < 0.05$) follicle diameter in the 10 mIU/mL pFSH treatment than in the control group. When comparing follicle categories within the same treatment (i.e., same concentration), although antral follicles were larger ($P < 0.05$) than preantral follicles at the onset of culture, the diameter of the two follicle types was similar ($P > 0.05$) in the control and pFSH10 treatment groups at the end of the culture period. Also, pFSH10 (for antral follicles) and pFSH100 (for both preantral and antral follicles) treatments resulted in a lesser ($P < 0.05$) daily growth rate of follicles compared to follicle growth rate in the control group (Fig. 3).

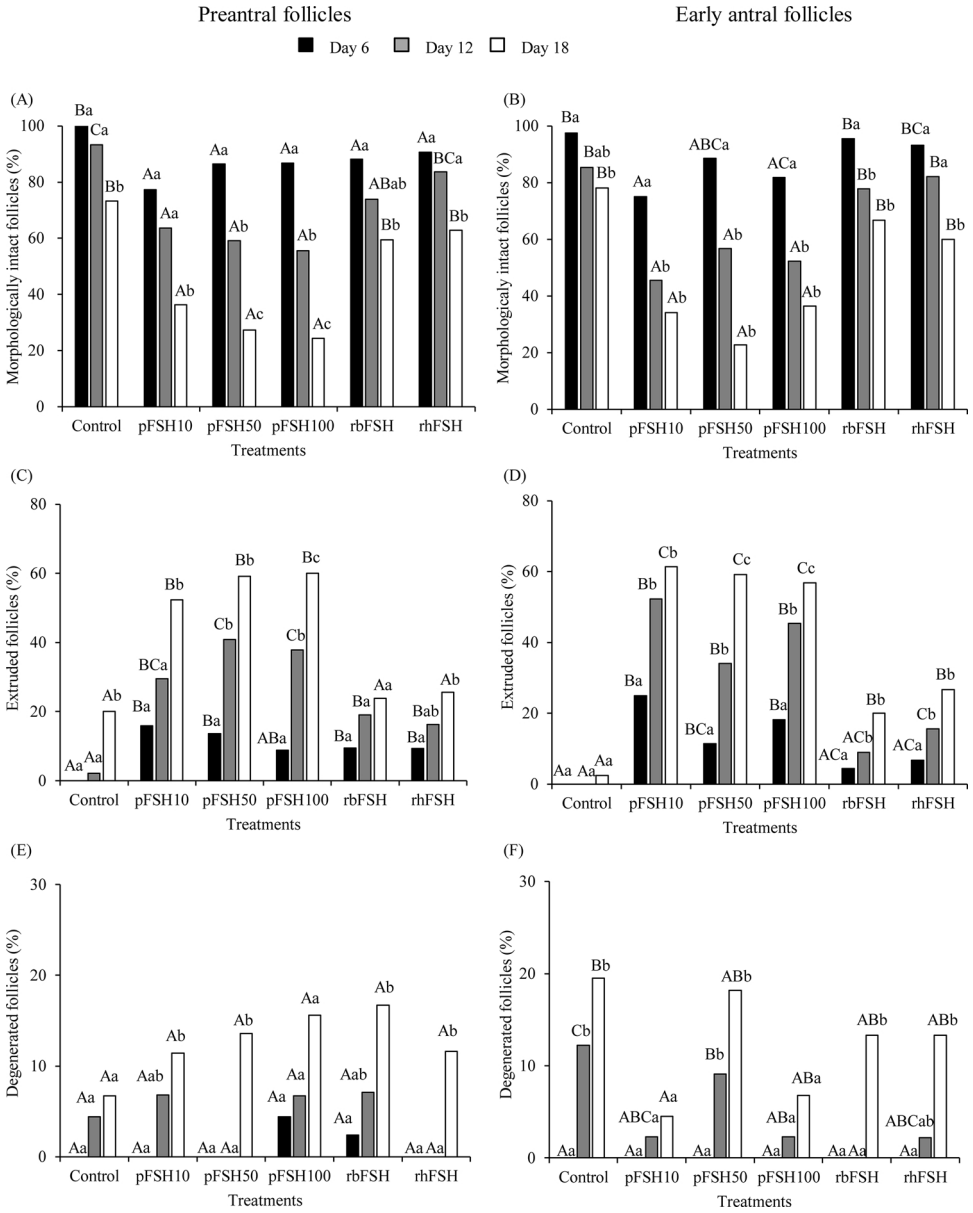


**Fig. 1.** Percentage of morphologically (A,B) intact, (C,D) extruded, and (E,F) degenerated preantral and early antral follicles of goats, respectively, after *in vitro* culture in the absence (control) or presence of pFSH (10, 50, or 100 mIU/mL), rbFSH (100 ng/mL), and rhFSH (50 mIU/mL) for 6, 12, and 18 days; $^{A–C}$ Values without a common letter indicate a difference among treatments when there was the same end point and follicle category ($P < 0.05$); $^{a–c}$ Values without a common letter indicate a difference among days within the same treatment group and follicle category ($P < 0.05$).

### *3.2. In vitro maturation and chromatin configuration of oocytes from preantral and early antral follicles grown in vitro*

After *in vitro* maturation, the mean diameter of the recovered viable oocytes did not differ ($P > 0.05$) from the control group within each follicle category (Table 3). In general, the mean diameter of viable oocytes was larger ($P < 0.05$) in antral than preantral follicles within the same treatment group. Only in antral follicles, the rhFSH treatment induced development of a larger ($P < 0.05$) oocyte diameter than the rbFSH treatment. Except for the pFSH100 (preantral follicles) and control (antral follicles) treatments, all the other treatments resulted in development of viable oocytes ≥120 μm by the end of the culture period.

The percentages of degenerated, germinal vesicle, meiotic resumption (including germinal vesicle breakdown, and MI and MII oocytes), and metaphase II (MII) oocytes after IVM are included in Table 4. Interestingly, the two types of recombinant FSH differentially affected antral and preantral follicle development. When there was treatment with rbFSH there were greater ($P < 0.05$) degeneration rates of preantral follicles compared with follicle degeneration rates in the control group. The addition of pFSH10, pFSH100, and rhFSH resulted in a lesser ($P < 0.05$) oocyte meiotic resumption in preantral follicles. Except for

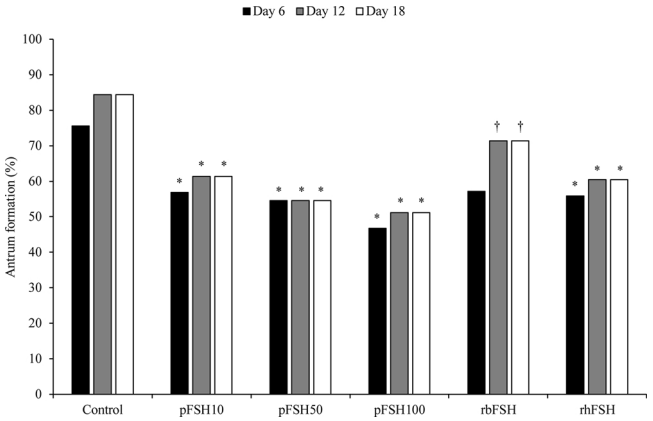


**Fig. 2.** Percentage of antrum formation of preantral follicles of goats cultured in the absence (control) or presence of pFSH (10, 50, or 100 mIU/mL), rbFSH (100 ng/mL), and rhFSH (50 mIU/mL) for 18 days; * Indicates a difference between the control and other treatment groups within the same days of culture ($P < 0.05$); † Indicates a difference in response to pFSH100 and rbFSH during the same days of the culture period ($P < 0.05$).

**Table 2**
Mean ( ± SEM) diameter (μm) of preantral and early antral follicles of goats cultured without (control) or with pFSH (10, 50, or 100 mIU/mL), rbFSH (100 ng/mL), and rhFSH (50 mIU/mL) for 18 days.

| Treatments | Day 0 | Day 6 | Day 12 | Day 18 |
|---|---|---|---|---|
| Preantral follicles | | | | |
| Control | 255.9 ± 3.4$^{Aa}$ | 393.6 ± 10.2$^{Bb}$ | 527.6 ± 20.4$^{Bc}$ | 621.6 ± 37.2$^{Cd}$ |
| pFSH10 | 251.2 ± 4.2$^{Aa}$ | 393.7 ± 13.8$^{Bb}$ | 484.2 ± 15.6$^{Abc}$ | 520.6 ± 19.2$^{ABc}$ |
| pFSH50 | 255.1 ± 4.4$^{Aa}$ | 380.3 ± 13.5$^{ABb}$ | 440.7 ± 20.2$^{Ac}$ | 484.6 ± 33.1$^{Ac}$ |
| pFSH100 | 254.0 ± 5.7$^{Aa}$ | 346.2 ± 10.7$^{Ab}$ | 448.1 ± 18.0$^{Ac}$ | 484.2 ± 29.3$^{Ac}$ |
| rbFSH | 251.7 ± 4.4$^{Aa}$ | 354.5 ± 14.6$^{Ab}$ | 499.1 ± 30.1$^{Abc}$ | 588.2 ± 39.2$^{ABCd}$ |
| rhFSH | 251.4 ± 5.2$^{Aa}$ | 358.3 ± 11.6$^{ABb}$ | 488.3 ± 23.0$^{Abc}$ | 602.5 ± 27.7$^{BCd}$ |
| Antral follicles | | | | |
| Control | 343.8 ± 4.8$^{Aa*}$ | 477.1 ± 11.2$^{Ab*}$ | 633.9 ± 26.3$^{Ac*}$ | 762.5 ± 43.5$^{Ad}$ |
| pFSH10 | 341.3 ± 6.0$^{Aa*}$ | 457.0 ± 15.6$^{Ab*}$ | 556.0 ± 18.0$^{Ac}$ | 618.0 ± 27.9$^{Bc}$ |
| pFSH50 | 344.5 ± 5.3$^{Aa*}$ | 468.8 ± 11.8$^{Ab*}$ | 577.1 ± 24.6$^{Ac*}$ | 661.0 ± 54.3$^{ABc*}$ |
| pFSH100 | 342.6 ± 5.8$^{Aa*}$ | 467.9 ± 12.0$^{Ab*}$ | 543.5 ± 19.5$^{Ac*}$ | 626.5 ± 25.9$^{ABd*}$ |
| rbFSH | 341.0 ± 5.2$^{Aa*}$ | 457.0 ± 12.2$^{Ab*}$ | 606.4 ± 22.6$^{Ac*}$ | 702.3 ± 39.2$^{ABd*}$ |
| rhFSH | 344.0 ± 5.4$^{Aa*}$ | 450.9 ± 10.7$^{Ab*}$ | 604.2 ± 20.8$^{Ac\ *}$ | 742.4 ± 34.0$^{Ad*}$ |

$^{A–C}$ Within a column, values without a common letter indicate a difference among treatments within the same days and follicle category ($P < 0.05$).
$^{a–d}$ Within a row, values without a common letter indicate a difference among days within the same treatment ($P < 0.05$).
* Indicates a difference between preantral and antral follicles within the same treatment and day of culture ($P < 0.05$).

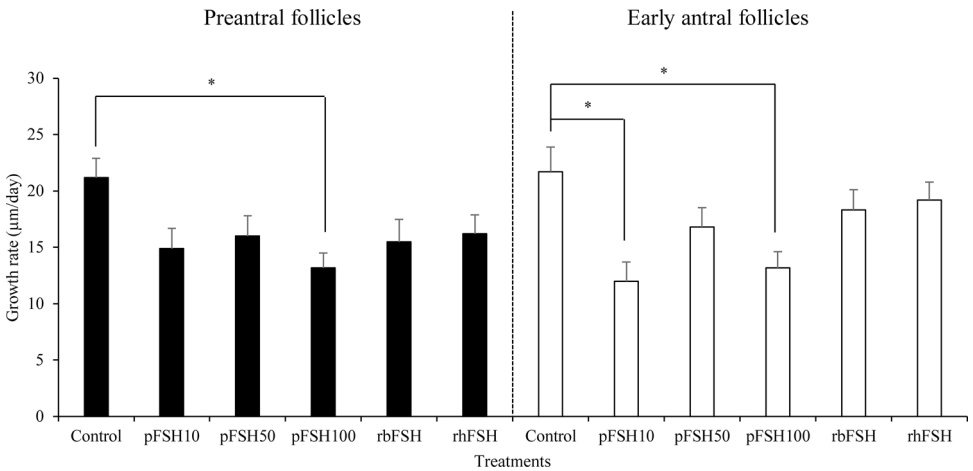


**Fig. 3.** Mean ( ± SEM) growth rate of preantral and early antral follicles of goats cultured without (control) or with addition of pFSH (10, 50, or 100 mIU/mL), rbFSH (100 ng/mL), and rhFSH (50 mIU/mL) for 18 days; *Indicates a difference ($P < 0.05$) between two treatments within the same follicle category.

**Table 3**
Mean ( ± SEM) diameter of viable oocytes, and percentages of fully-developed oocytes from preantral and early antral follicles of goats cultured without (control) or with addition of pFSH (10, 50, or 100 mIU/mL), rbFSH (100 ng/mL), and rhFSH (50 mIU/mL) for 18 days.

| Treatments | Overall diameter of viable oocytes | Oocytes ≥110 μm (%)† | Diameter of oocytes ≥110 μm | Oocytes ≥120 μm (%)† | Diameter of oocytes ≥120 μm |
|---|---|---|---|---|---|
| Preantral follicles | | | | | |
| Control | 95.8 ± 2.0 | 11.1 (5/45) | 117.6 ± 0.8 | 2.2 (1/45) | 120.2 ± 0.0 |
| pFSH10 | 90.3 ± 2.0 | 4.5 (2/44) | 119.5 ± 7.1 | 2.3 (1/44) | 126.5 ± 0.0 |
| pFSH50 | 93.9 ± 2.6 | 15.9 (7/44) | 118.3 ± 2.5 | 6.8 (3/44) | 124.2 ± 2.0 |
| pFSH100 | 89.4 ± 1.9 | 4.4 (2/45) | 113.6 ± 2.0 | 0.0 (0/45) | 0.0 ± 0.0 |
| rbFSH | 97.4 ± 2.4 | 16.7 (7/42) | 115.5 ± 1.8 | 4.8 (2/42) | 121.8 ± 0.3 |
| rhFSH | 92.7 ± 1.7 | 4.6 (2/43) | 118.5 ± 5.1 | 2.3 (1/43) | 123.6 ± 0.0 |
| Antral follicles | | | | | |
| Control | 103.9 ± 1.9$^{AB,*}$ | 29.3 (12/41)* | 112.8 ± 1.2 | 0.0 (0/41)$^{A}$ | 0.0 ± 0.0 |
| pFSH10 | 103.3 ± 2.2$^{AB,*}$ | 27.3 (12/44)* | 118.7 ± 1.0 | 11.4 (5/44)$^{B}$ | 121.7 ± 0.5 |
| pFSH50 | 103.4 ± 2.4$^{AB,*}$ | 13.6 (6/44) | 122.0 ± 2.2 | 9.1 (4/44)$^{AB}$ | 125.0 ± 0.8 |
| pFSH100 | 105.4 ± 2.1$^{AB,*}$ | 27.3 (12/44)* | 117.9 ± 1.9 | 11.4 (5/44)$^{B*}$ | 124.2 ± 0.7 |
| rbFSH | 99.3 ± 2.2$^{A}$ | 15.6 (7/45) | 116.7 ± 2.1 | 4.4 (2/45)$^{AB}$ | 124.0 ± 1.1 |
| rhFSH | 108.9 ± 2.3$^{B*}$ | 28.9 (13/45)* | 118.1 ± 1.6 | 11.1 (5/45)$^{B}$ | 123.7 ± 0.9 |

$^{AB}$ Values without a common letter indicate a difference among treatments within the same follicle category ($P < 0.05$).
* Indicates a difference between preantral and antral follicles within the same treatment ($P < 0.05$).
† Percentage of fully-developed oocytes for each treatment was calculated in relation to the total number of follicles present at the initiation of the culture period.

**Table 4**
Percentages of degenerated oocytes, germinal vesicle stage (GV), meiotic resumption, and MII, after the IVM of oocytes from preantral and early antral follicles of goats cultured without (control) or with addition of pFSH (10, 50, or 100 mIU/mL), rbFSH (100 ng/mL), and rhFSH (50 mIU/mL) for 18 days.

| Treatments | Degenerated oocytes (%) | Germinal vesicle (%) | Meiotic resumption (%)† | MII (%) |
|---|---|---|---|---|
| Preantral follicles | | | | |
| Control | 11.1 (5/45)$^{A}$ | 62.2 (28/45)$^{AB}$ | 26.7 (12/45)$^{B}$ | 13.3 (6/45)$^{A}$ |
| pFSH10 | 22.7 (10/44)$^{AB}$ | 68.2 (30/44)$^{A}$ | 9.1 (4/44)$^{A}$ | 4.5 (2/44)$^{A}$ |
| pFSH50 | 20.4 (9/44)$^{AB}$ | 61.4 (27/44)$^{AB}$ | 18.2 (8/44)$^{AB}$ | 15.9 (7/44)$^{A}$ |
| pFSH100 | 20.0 (9/45)$^{AB}$ | 73.3 (33/45)$^{A}$ | 6.7 (3/45)$^{A}$ | 0.0 (0/45)$^{B}$ |
| rbFSH | 30.9 (13/42)$^{B}$ | 42.8 (18/42)$^{B}$ | 26.2 (11/42)$^{B}$ | 11.9 (5/42)$^{A}$ |
| rhFSH | 18.6 (8/43)$^{AB}$ | 72.1 (31/43)$^{A}$ | 9.3 (4/43)$^{A}$ | 0.0 (0/43)$^{B}$ |
| Antral follicles | | | | |
| Control | 26.8 (11/41) | 31.7 (13/41)$^{AB*}$ | 41.5 (17/41)$^{A}$ | 7.3 (3/41)$^{A‡}$ |
| pFSH10 | 27.3 (10/44) | 47.7 (21/44)$^{B*}$ | 29.5 (13/44)$^{A*}$ | 20.5 (9/44)$^{BC}$ |
| pFSH50 | 40.9 (18/44)* | 36.4 (16/44)$^{AB*}$ | 27.3 (10/44)$^{A}$ | 9.1 (4/44)$^{AB\#}$ |
| pFSH100 | 29.5 (13/44) | 36.4 (16/44)$^{AB*}$ | 34.1 (15/44)$^{A*}$ | 18.2 (8/44)$^{ABC}$ |
| rbFSH | 31.1 (14/45) | 42.2 (19/45)$^{B}$ | 26.7 (12/45)$^{A}$ | 0.0 (0/45)$^{A}$ |
| rhFSH | 42.2 (19/45)* | 22.2 (10/45)$^{A*}$ | 35.5 (16/45)$^{A*}$ | 22.2 (10/45)$^{C}$ |

$^{A–C}$ Values without a common letter indicate a difference among treatments within the same end points and follicle category ($P < 0.05$).
* Indicates a difference between preantral and antral follicles within the same treatment ($P < 0.05$).
† Included germinal vesicle breakdown and metaphases I (MI) and II (MII) oocytes.
‡ Tended to differ from pFSH10 within antral follicles ($P = 0.08$).
# Tended to differ from rhFSH within antral follicles ($P = 0.08$).

pFSH100, however, the inclusion in the media of the other two concentrations evaluated (pFSH10 and 50) resulted in development of MII oocytes in cultured preantral follicles. Treatments with rhFSH did not result in development of any MII oocytes in preantral follicles. Nonetheless, rbFSH was the only treatment that did not affect oocyte nuclear maturation in antral follicles. The addition of rhFSH and pFSH10 resulted in an increase ($P = 0.08 – P < 0.05$) in the percentage of MII oocytes from antral follicles compared to the control group. Also, oocyte maturation tended ($P = 0.08$) to be greater in the rhFSH treatment than the pFSH50 treatment group.

### *3.3. Relationships among follicle morphology, antrum formation, oocyte viability, and chromatin configuration*

Based on the data from both individual follicle culture and individual IVM, a multiple logistic regression analysis was performed to evaluate the effects of follicle morphology (intact, extruded, or degenerated) and antrum formation (only in preantral follicles) on oocyte viability, meiotic resumption, and meiotic competence (MII) (Fig. 4). Antrum formation was positively associated with oocyte viability (OR = 6.5, $P < 0.001$), meiotic resumption (OR = 3.3, $P < 0.007$), and meiotic competence (OR = 3.4, $P = 0.06$). Furthermore, follicle extrusion in both follicular categories was positively correlated with oocyte viability (preantral follicles, OR =

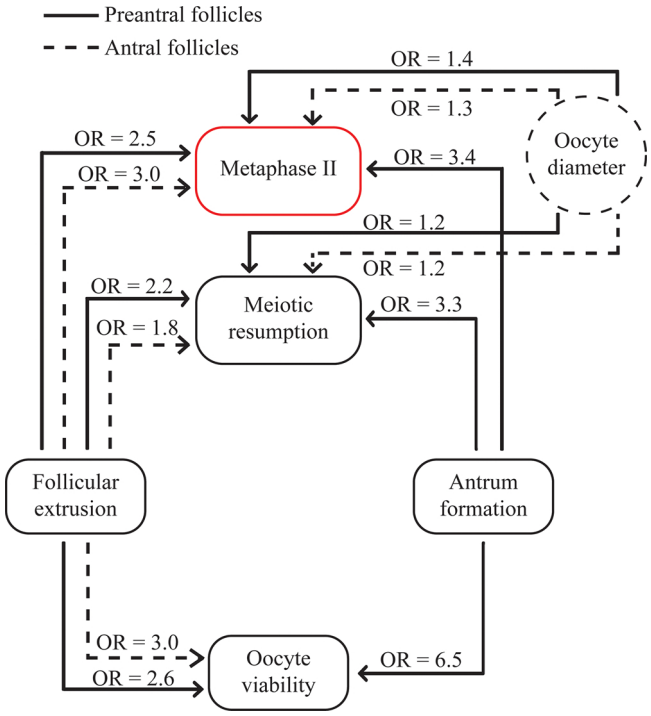


**Fig. 4.** Diagram depicting novel and important positive relationships detected by logistic regression analyses (odds ratio, OR) among several follicle and oocyte end points; Regardless of treatment, all oocytes were used for the analyses; Arrows represent the effect of one variable on another variable; OR number represents how many times there was a greater chance of a specific variable having a specific effect on another variable (e.g., in a follicle that forms an antrum there is a 6.5 times greater probability of there being development of a viable oocyte).

2.6; antral follicles, OR = 3.0; $P < 0.001$), meiotic resumption (preantral follicles, OR = 2.2, $P < 0.001$; antral follicles, OR = 1.8, $P < 0.007$) and meiotic competence (preantral follicles, OR = 2.5, $P < 0.04$; antral follicles, OR = 3.0, $P < 0.001$). In addition, the oocyte diameter in both follicular categories was positively correlated with meiotic resumption (preantral follicles, OR = 1.2, $P < 0.001$; antral follicles, OR = 1.2, $P < 0.001$), and completion of methaphase II (preantral follicles, OR = 1.4, $P < 0.001$; antral follicles, OR = 1.3, $P < 0.001$).

### *3.4. Relative abundance of mRNA transcripts in follicular walls*

The values for relative abundance of *MMP-9, TIMP-2, CYP17, CYP19A1, FSHR*, *Insulin-R* mRNA transcripts and *BAX/BCL-2* transcript ratio are depicted in Fig. 5. The *MMP-9* mRNA transcript was not detected in preantral or antral follicles of the control group or in preantral follicles of the rhFSH treatment group (Fig. 5A). Nevertheless, the relative abundance of *MMP-9* mRNA transcript was greater ($P < 0.05$) for preantral follicles in the pFSH100 treatment compared with the rbFSH treatment group. For antral follicles, however, the relative abundance of *MMP-9* mRNA transcript was greater ($P < 0.05$) with the pFSH10 and pFSH50 treatments than rbFSH and rhFSH treatments. The relative abundance of *TIMP-2* mRNA transcript in preantral follicle walls was lesser ($P < 0.05$) with the pFSH50 treatment than in the control, rbFSH, and rhFSH treatment groups (Fig. 5B). For preantral follicles, with all treatments other than pFSH there was a larger ($P < 0.05$) relative abundance of *CYP17* mRNA transcript than when there were treatments containing pituitary FSH (pFSH10, pFSH50, and pFSH100; Fig. 5C). Nevertheless, the control group had the largest ($P < 0.05$) relative abundance of *CYP17* mRNA transcript among all treatment groups. For antral follicles, the relative abundance of *CYP17* transcript in the control group was larger ($P < 0.05$) than in pFSH50 and pFSH100 treatment groups. The relative abundance of *CYP19A1* mRNA transcript in preantral follicles was greater ($P < 0.05$) in the pFSH50 and pFSH100 treatment groups than in control, rbFSH, and rhFSH treatment groups, although it did not differ from that of the pFSH10 treatment group (Fig. 5D). For antral follicles, the relative abundance of *CYP19A1* mRNA transcript was greater ($P < 0.05$) in the pFSH100 than all other treatment groups, except for the pFSH10 group. The relative abundance of *FSHR* mRNA transcript in preantral follicles was similar ($P > 0.05$; Fig. 5E); however, in antral follicles, relative abundance of *FSHR* mRNA transcript was greater ($P < 0.05$) in the pFSH100 compared to the pFSH50 treatment groups. There was a much larger ($P < 0.05$) relative abundance of *Insulin-R* mRNA transcript in preantral follicles in the control and rhFSH than pFSH50 and pFSH100 treatment groups (Fig. 5F). For antral follicles, the relative abundance of *Insulin-R* mRNA transcript was larger ($P < 0.05$) in rbFSH compared with all other treatment groups when there was culturing with pFSH. Furthermore, when comparing preantral to antral follicles within the same treatment group, the relative abundance of *Insulin-R* in pFSH50, rbFSH, and rhFSH treatment groups was less ($P < 0.05$) in preantral follicles. The *BAX/BCL2* ratio in preantral and early antral follicles was similar among treatment groups (Fig. 5G).

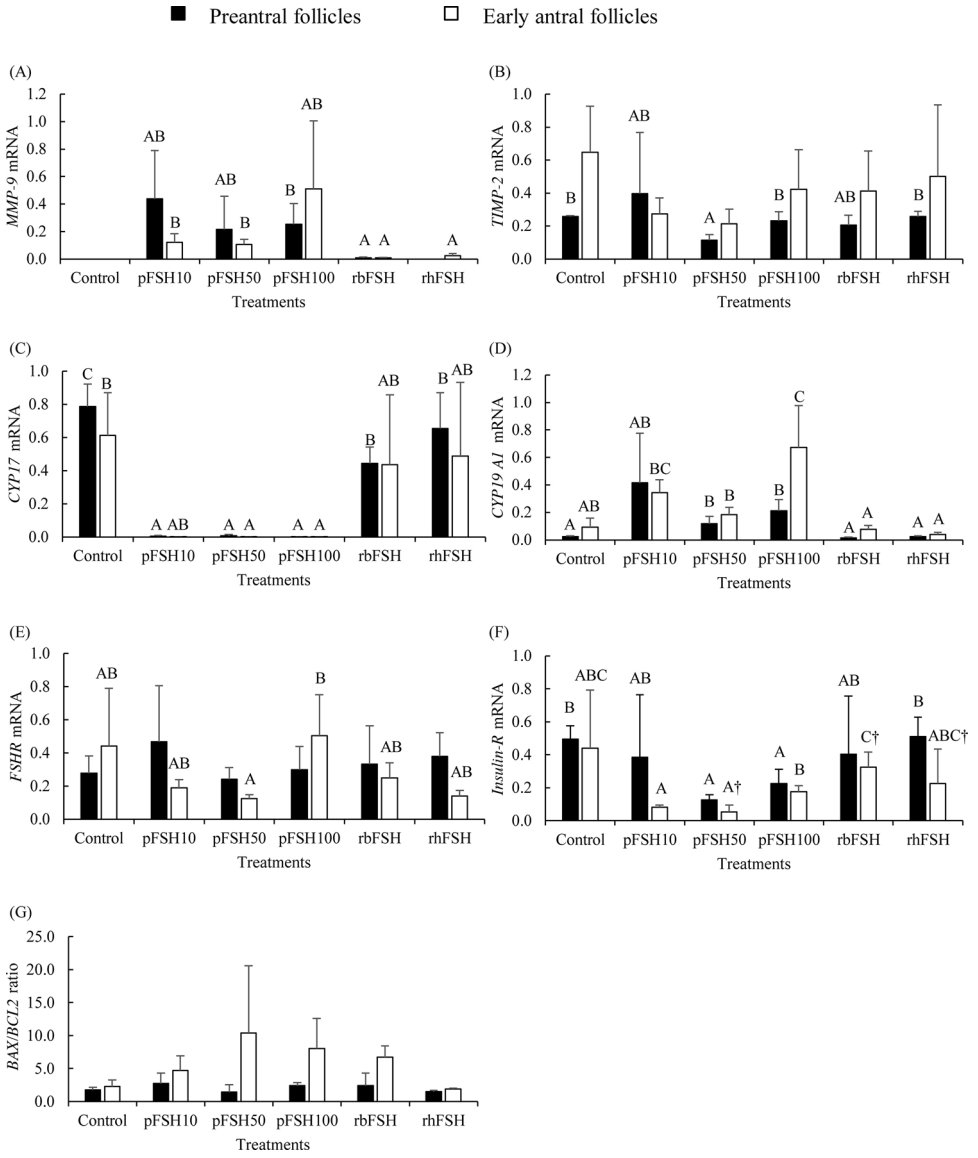


**Fig. 5.** Mean ( ± SEM) relative abundance of (A) *MMP-9*, (B) *TIMP-2*, (C) *CYP17*, (D) *CYP19A1*, (E) *FSHR*, (F) *Insulin-R* mRNA transcripts and (G) *BAX/BCL-2* mRNA transcript ratio in preantral and early antral follicles of goats cultured without (control) or with addition of FSH for 18 days; [A–C]Values without a common letter indicate a difference among treatments within the same end point and follicle category ($P < 0.05$). † Indicates a difference between preantral and antral follicles within the same treatment group ($P < 0.05$).

### 3.5. Estradiol production

Estradiol concentrations were individually quantified in conditioned media only from follicles containing oocytes that resumed meiosis (MI and MII) after 18 days of culture (Table 5). Regardless of the follicle category, the addition of pituitary FSH led to a reduction ($P < 0.05$) in estradiol production. Furthermore, with the rbFSH treatment there was a lesser ($P < 0.05$) estradiol concentration after preantral follicle culture than with the control group. When estradiol production was compared for antral and preantral follicles, the only difference was the greater ($P < 0.05$) concentration after the culture of antral follicles with rbFSH.

## 4. Discussion

The present study was conducted to compare, for the first time, the effect of different concentrations and sources of FSH (pFSH, rbFSH, and rhFSH) on the *in vitro* development of two follicle categories (preantral and antral follicles) when there were the same experimental conditions. Results from the present study indicate that different sources of FSH differentially affect follicle development *in vitro*, including follicle growth, antrum formation, estradiol production, oocyte meiotic maturation, and relative abundance of

**Table 5**
Mean ( ± SEM) estradiol concentrations on day 18 of *in vitro* culture of preantral and early antral follicles of goats cultured without (control) or with additions of pFSH (10, 50, or 100 mIU/mL), rbFSH (100 ng/mL), and rhFSH (50 mIU/mL).

| Treatments | Concentration (ng/mL) |
|---|---|
| Preantral follicles | |
| Control | $43.3 \pm 5.9^{C}$ |
| pFSH10 | $3.3 \pm 0.1^{A}$ |
| pFSH50 | $2.0 \pm 0.3^{A}$ |
| pFSH100 | $2.1 \pm 0.5^{A}$ |
| rbFSH | $16.3 \pm 2.2^{B}$ |
| rhFSH | $57.3 \pm 17.5^{BC}$ |
| Antral follicles | |
| Control | $67.7 \pm 22.0^{B}$ |
| pFSH10 | $2.7 \pm 0.8^{A}$ |
| pFSH50 | $2.3 \pm 0.5^{A}$ |
| pFSH100 | $2.1 \pm 0.5^{A}$ |
| rbFSH | $52.2 \pm 2.9^{B}$* |
| rhFSH | $68.9 \pm 14.4^{B}$ |

[AB] Values without a common letter indicate difference among treatments within the same follicle category ($P < 0.05$).
*Indicates a difference between preantral and antral follicles within the same treatment ($P < 0.05$).

*MMP-9, TIMP-2*, *FSHR*, *Insulin-R*, and *CYP17* and *CYP19A1* (steroidogenic enzymes) mRNA transcripts. Furthermore, preantral as compared with antral follicles can respond differently to the same source/concentration of FSH. In addition, as a secondary and novel finding in the present study, regardless of treatment, there were important positive relationships among the values for several follicle and oocyte end points when there was use of logistic regression analyses for these evaluations (odds ratio).

A great variety of FSH sources has been used for the *in vitro* culture of ovarian follicles from different species, such as pituitary (goat, Magalhães et al., 2009a), recombinant (goat: Ferreira et al., 2018; sheep: Barros et al., 2019; horse: Aguiar et al., 2016; cattle: McLaughlin and Telfer, 2010; mouse: Adriaens et al., 2004; monkey: Xu et al., 2010; dog: Serafim et al., 2013), and urinary (mouse: Park et al., 2013). Furthermore, heterogeneous concentrations of FSH have been used, which commonly range from 1 to 2000 mIU/mL (Naydu and Osborn, 1992; Ihm et al., 2015) or 50–1000 ng/mL (Saraiva et al., 2011). In the present study, when there was use of pFSH there was a lesser percentage of morphologically intact follicles and concomitantly increased follicle extrusion. Magalhães et al. (2009a) reported that recombinant FSH (bovine) and pituitary FSH (porcine) differentially affected the *in vitro* development of preantral follicles (especially primordial follicles) contained in ovarian tissue fragments of goats. The elevated extrusion rates observed when there were treatments with pFSH could be due to the extent of purity and/or differences in molecular structure. Unlike recombinant FSH, with a relatively greater purity, pituitary FSH may contain different pituitary impurities, such as LH (Closset and Hennen, 1989), affecting its bioactivity. In addition, the purification process can alter the isoforms of this hormone (Bousfield et al., 2008). At least 15 different FSH isoforms are known to be secreted by the pituitary gland, all of these being physiologically and functionally different (Stanton et al., 1996; Timossi et al., 2000; Creus et al., 1996). Changes in molecular weight, plasma half-life, isoelectric properties, and bioactivity are the result of carbohydrate variations within the oligosaccharides linked to the different FSH isoforms (Andersen et al., 2001; Ayres et al., 2018). By removing these oligosaccharides, there is a progressive loss of the affinity of FSH for the respective receptor and thereby lesser bioactivity (Ryan et al., 1988; Walton et al., 2001). Different commercially available pituitary FSH preparations (Stimufol® and Folltropin®) have an effect on goat follicle viability and growth *in vitro* differentially, probably due to the different FSH:LH ratio, that is 20:1 for Stimufol® and 5.25:1 for Folltropin® (Magalhães et al., 2009b).

Besides the differences between pituitary and recombinant FSH for some end points, differences were also observed between the two types of recombinant FSH (bovine or human), in oocyte diameter for antral follicles, meiotic resumption for preantral follicles, and percentage of MII oocytes in both follicle categories evaluated in the present study. Small differences in the complex molecular structure of the FSH types, along with the differences in the concentrations used, could explain the variation in findings in the present study. In this regard, differences in the glycosylation profile of two commercial recombinant human FSH preparations (Bemfola and GONAL-f) have an effect on the bioactivity of these preparations (Mastrangeli et al., 2017). Furthermore, in a study on the differential thermal stability of recombinant human, bovine, and ovine FSH there was less stability in the quaternary structure of bovine FSH compared with that of human and ovine FSH (Hassan et al., 2015).

Preantral and early antral follicles responded differently to the same culture conditions in the present study. For example, the addition of rhFSH to the culture media led to greater oocyte maturation in antral follicles, while there was no development of MII oocytes in preantral follicles, and the opposite was observed when rbFSH was added to the media. Furthermore, the addition of 50 mIU/mL pFSH to the media led to greater maturation rates in preantral follicles than in antral follicles. In this regard, from previous studies there have also been reported differences in treatment responses between preantral and antral follicles of goats *in vitro* when there were treatments with growth hormone (Cadenas et al., 2017) or increasing concentrations of rhFSH (Ferreira et al., 2018). In the mouse, preantral follicles responded to FSH in a size-dependent manner (Hardy et al., 2017). Results from studies focused on gene

expression patterns indicate there are differences in responses between preantral and antral follicles when there are the same culture conditions. There are approximately 2466 genes expressed in a follicle stage-specific manner being up- or down-regulated in the transition between secondary and early tertiary follicle developmental stages (Magalhães-Padilha et al., 2013).

In conclusion, the source of FSH affects gene expression and follicle dynamics of preantral and early antral follicles *in vitro* differentially. Even though there is this difference, the addition of this gonadotropin to the *in vitro* culture of preantral follicles of goats did not improve the overall outcome. Nonetheless, either treatment with pFSH10 or rhFSH enhanced the meiotic competence of oocytes from antral follicles of goats during the early developmental stages. Furthermore, the results of the present study indicate there are novel and important positive relationships among several follicle and oocyte end points, regardless of treatment.

## Author's contributions

The main experimental conception and design were created by A.C.A.F., B.G.A., L.F.L., J.R.F. All laboratory procedures were carried out by A.C.A.F, N.A.R.S., J.C., H.H.V.C., and D.D.G. Contributed reagents/materials/analysis tools: J.R.F., A.P.R.R., and J.J.H.C. Data collection, statistical analyses, and interpretation were performed by A.C.A.F., B.G.A., J.J.H.C., E.L.G., and J.R.F. Manuscript writing and proofreading A.C.A.F., J.C., B.G.A., L.F.L., E.L.G., A.P.R.R., and J.R.F. All authors have read and approved the final manuscript.

## Declaration of Competing Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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