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Accelerated follicle growth during the culture of isolated caprine preantral follicles is detrimental to follicular survival and oocyte meiotic resumption

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ABSTRACT

This study investigated the effect of androstenedione (A4) alone or in association with different concentrations of bovine recombinant FSH on the IVC of isolated goat preantral follicles. Follicles were mechanically isolated from ovarian tissue and cultured for 18 days in α–minimum essential medium supplemented or not with A4 (10 ng/mL) alone or in association with fixed (A4 + FixFSH: 100 ng/mL) or sequential (A4 + SeqFSH: Day 0, 100 ng/mL; Day 6, 500 ng/mL; Day 12, 1000 ng/mL) concentrations of FSH. After 18 days, the oocytes were recovered for IVM and fluorescence analysis. At Day 18 of culture, only A4 + SeqFSH treatment showed a lower ($P < 0.05$) rate of intact follicles, survival probability, and meiotic resumption, as well as higher ($P < 0.05$) percentage of degeneration and/or extrusion after antrum formation. Taken together, these results reported a positive correlation between fast-growing follicles and follicles that degenerated and/or extruded after antrum formation. When compared with control, the addition of A4 alone or in association of FSH did not increase ($P > 0.05$) the estradiol production or androstenedione levels on Day 6. However, on Day 18, the androstenedione levels were significantly lower in A4 + SeqFSH treatment when compared with A4 alone or to A4 + FixFSH treatments, whereas the estradiol production did not differ ($P > 0.05$). In summary, this study found that accelerated follicle growth negatively impacted the morphology of caprine preantral follicle cultured *in vitro*. In addition, the association of androstenedione with increasing concentration of FSH was detrimental to follicular survival and oocyte meiotic resumption.
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1. Introduction

Ovarian function depends on multiple integrated processes that culminate in the production of competent oocytes during folliculogenesis. FSH plays an important role in folliculogenesis [1]. Regardless the FSH addition

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(i.e., fixed or increased concentrations) in IVC media [2,3], it provides for the maintenance of follicular growth and survival as well as stimulates steroidogenesis [4]. Among the steroidal hormones, addition of androgens (androstenedione, dihidroandrostenediona, dihydrotestosterone, and testosterone) promotes follicular growth in different species [5–7] through the stimulation of their receptors. Androgen receptors are located in the cytoplasm of granulosa cells (GC), theca cells (TC), and oocytes [8] of preantral follicles [9–11]; and antral [9,10,12], being the GC more responsive to androgens [13].

An important tool to investigate *in vitro* folliculogenesis, including the effect of different substances, is the IVC of preantral follicles [14]. Studies evaluating the role of androstenedione on *in vitro* follicle culture have been controversial with findings being both concentration-dependent and species specific. For example, addition of androstenedione in a dose-dependent manner reduced the survival rate of isolated mice preantral follicles [15]. On the other hand, culture of isolated swine preantral follicles in medium containing androstenedione improved antrum formation rates [16]. In another study, after IVC of caprine preantral follicles (primordial and primary) enclosed in ovarian tissue, androstenedione associated with fixed FSH maintained the percentage of normal follicles during the culture [17]. However, the effect of androstenedione and FSH on IVC of isolated secondary preantral follicles has not been fully investigated in caprines. It is well known that goats are spread worldwide, having social and economic importance in many countries. In addition, the goat has been proposed as an important animal model for women [18].

Therefore, the objective of this study was to investigate the effect of androstenedione addition, associated or not with FSH (fixed or sequential concentration) into the culture medium, by evaluating the following end points: (1) follicular survival and growth, (2) antrum formation, (3) hormone production (androstenedione and estradiol) of caprine isolated secondary follicles, and (4) oocyte meiotic resumption.

2. Material and methods

2.1. Chemicals

Unless stated otherwise, all the chemicals and culture media used for this study were purchased from Sigma Chemical Company (St. Louis, MO, USA).

2.2. Source of ovaries

Ovaries (n = 60) from 30 adults (between 1 and 3 years old), nonpregnant, mixed-breed goats (*Capra hircus*) were collected at a slaughterhouse. After slaughter, the pairs of ovaries were washed in 70% alcohol for 10 seconds and then twice in minimum-essential-medium plus HEPES (MEM-HEPES) supplemented with 100 μg/mL penicillin and 100 μg/mL streptomycin. After which, the ovaries were transported within 4 hours to the laboratory in MEM-HEPES at 4 °C [19].

2.3. Isolation and selection of preantral follicles

In the laboratory, the surrounding fatty tissue and ligaments were stripped from the ovaries. Ovarian cortical slices (1- to 2-mm thick) were excised from the ovarian surface using a surgical blade under sterile conditions and subsequently placed in holding medium consisting of MEM-HEPES with antibiotics. Preantral follicles, approximately 150–200 μm in diameter without antral cavities (secondary follicles), were visualized under a stereomicroscope (model SMZ 645; Nikon, Tokyo, Japan) and mechanically isolated by using 26-gauge needles. Approximately 10 secondary follicles were isolated from each ovarian pair. These follicles were then transferred to 100-μL droplets containing basic culture medium for the evaluation of quality. Only secondary follicles that displayed the following characteristics were selected for culture: an intact basement membrane, two or more layers of GCs, and a visible oocyte that was round and centrally located within the follicle, without any dark cytoplasm. Then, isolated follicles were pooled and randomly allocated to the treatments [20].

2.4. IVC of preantral follicles

After selection, the follicles were individually cultured (one follicle per droplet) in 100-μL droplets of culture medium under mineral oil in Petri dishes (60 × 15 mm; Corning, Sarstedt, Newton, NC, USA). The medium used was α-MEM (pH 7.2–7.4; Gibco, Invitrogen, Karlsruhe, Germany) supplemented with 3.0 mg/mL BSA, 10 ng/mL insulin, 5.5 μg/mL transferrin, 5 ng/mL selenium, 2-mM glutamine, 2-mM hypoxanthine, and 50 μg/mL ascorbic acid, here in after referred as α-MEM$^+$ [20]. Incubation was conducted at 39 °C and 5% CO_2 in air for 18 days. Fresh media were prepared and incubated for 1 hour before use, with 60-μL medium being changed in each drop every 2 days. The experimental conditions were replicated 4 times and at least 40 follicles were used per treatment.

2.5. Experimental design

Follicles were randomly assigned to 4 different treatments as follows: (1) α-MEM$^+$ alone (control group); (2) α-MEM$^+$ supplemented with androstenedione (A4: 10 ng/mL); (3) α-MEM$^+$ plus A4 (10 ng/mL) associated with recombinant bovine FSH, rbFSH (Nanocore, São Paulo, Brazil) in increasing concentrations (Sequential FSH: from Day 0 to Day 6 = 100 ng/mL; from Day 6 to Day 12 = 500 ng/mL; from Day 12 to Day 18 = 1000 ng/mL; A4 + SeqFSH); (4) α-MEM$^+$ plus A4 (10 ng/mL) associated with a fixed concentration of rbFSH (FixFSH: 100 ng/mL; A4 + FixFSH). Concentrations of A4 and rbFSH were chosen based on previous work performed by our group (A4: [17]; SeqFSH: [3]; FixFSH: [2]).

2.6. Morphologic evaluation of follicle development

Follicles were evaluated during culture (Days 0, 6, 12, and 18) to the following end points: integrity of the basement membrane, morphology of the oocyte and

surrounding GCs, and morphologic signs of degeneration such as darkness. The percentage of morphologically intact follicles was calculated by excluding the follicles which experienced rupture of the basement membrane and had degenerated. The percentage of degenerated follicles was calculated using the number of follicles that exhibited dark and/or misshapen cytoplasm of the oocyte and surrounding cumulus cells. The percentage of extruded follicles was then calculated using the number of follicles that reported rupture of the basement membrane. The follicular diameter was measured only in morphologically intact follicles every 6 days with the aid of an ocular micrometer attached to a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan; magnification: × 100). Two perpendicular diameters were recorded for each follicle, and the average of these 2 values was reported as the follicular diameter. To evaluate daily follicular growth, the mean increase in follicular diameter was calculated as follows: the diameter of morphologically intact follicles at Day 18 minus their diameter at Day 0, divided by 18 days. *In vitro* cultured follicles were divided into 3 growing categories: (1) nongrowing, follicles that did not grow during the culture; (2) slow growing, follicles with a daily growth between 0.1 and 7.0 μm/day; and (3) fast growing, follicles with a daily growth between 7.1 and 34 μm/day. Also, antral cavity formation was defined as a visible translucent cavity within the GC layers.

2.7. In vitro maturation

At the end of the culture period, all the morphologically intact 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 IVM. The oocyte recovery rate was calculated by the ratio between the number of oocytes ≥110 μm (including zona pellucida) and the total number of normal and degenerated oocytes (smaller and larger than 110 μm). The cumulus-oocyte complexes were selected and washed 3 times in maturation medium consisting of TCM-199 supplemented with 1% BSA, 5 μg/mL of LH, 0.5 μg/mL of rbFSH, 10 ng/mL of EGF, 50 ng/mL of recombinant insulin-like growth factor, 100 μM of cysteamine, 1 mM of pyruvate, and 1 μg/mL of estradiol [21]. After washing, oocytes were transferred to 100-μL drops of maturation medium under mineral oil and then incubated for 32 hours at 39 °C with 5% CO_2 in air.

2.8. Assessment of oocyte viability and chromatin configuration

Fluorescence microscopy was used to analyze the viability of the oocytes from the caprine follicles classified as morphologically normal under the stereomicroscope, after culture. Briefly, the oocytes were incubated in 4 μM calcein-AM, 2 μM ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany), 10 μM Hoechst 33342, and 0.5% glutaraldehyde (Sigma, Deisenhofen, Germany) at room temperature, for 30 minutes.

After the incubation, oocytes were washed 3 times in TCM 199 HEPES and placed in 3-μL droplets of mounting medium (6.25 mL of PBS, 6.25 mL of glycerol, and 6.25-μL Hoechst), covered with a cover slip on the slide and evaluated under a fluorescence microscope (Nikon, Eclipse 80i, Tokyo, Japan). Oocytes with cytoplasm stained by calcein-AM (green) were classified as viable and those that had chromatin marked with ethidium homodimer-1 (red) were considered to be degenerated. Hoechst was used to analyze the oocyte chromatin configuration for intact germinal vesicles, meiotic resumption (germinal-vesicle breakdown, metaphase I, and metaphase II).

2.9. Androstenedione and estradiol assay

To evaluate the relationship between follicle development and hormone production, 60 μL of culture medium from all the follicles was collected every 6 days (Days 6, 12, and 18) to quantify androstenedione (A4) and estradiol (E2) production. Androstenedione was measured by direct radioimmunoassay from Biosource (androstenedione-RIA-CT, BioSource Europe, Nivelles, Belgium) with a sensitivity of 70 ng/L and a total imprecision profile (coefficient of variation = 10%) for concentrations between 100 and 7000 ng/L. Hormone Assay for E2 (DSL4800) secretion was determined by double antibody radioimmunoassay (RIA) using a commercial kit Immunotech (catalog no: DSL4800, Immunotech s.r.o., Czech Republic). The analytical sensitivity of the assay was 2.2 pg/mL (assay range, 2.2–750 pg/mL). Intra-assay: samples were assayed 12 times in the same run. The coefficients of variation were ≤8.9%. Inter-assay coefficients of variation were assayed in duplicate within 8 different runs and were ≤12.2%. All samples were assayed in the same RIA to eliminate inter-assay variability.

2.10. Statistical analysis

All statistical analyses were performed using Sigma Plot 11 (Systat Software Inc., USA). Data that were not normally distributed (Shapiro–Wilk test) were submitted to logarithmic transformation. Follicle and oocyte diameter and follicular growth among treatments were compared by Kruskal–Wallis test, whereas the Wilcoxon signed test was used to analyze the effect of treatment within days of culture. The proportion of follicular variables (intact, degenerated, extruded, antrum formation, and meiotic resumption) among treatments and days of culture were analyzed by Fisher's exact test. A linear regression analysis was performed to evaluate the association of oocyte diameter with chromatin configuration and the relationship of follicular growth and antrum formation, whereas the logistic regression analyzed the association between follicle diameter and antrum formation. The follicle survival probability throughout the culture was plotted using Kaplan–Meier method, and the difference among survival curves (treatments) was evaluated using log rank test. Data are presented as mean (± standard error of mean) and percentage, and the results were considered different when $P < 0.05$. Probability values > 0.05 and ≤ 0.1 indicated that a difference approached significance.

3. Results

3.1. Follicular morphology

A total of 169 follicles were selected for IVC with a mean of 42.2 ± 0.6 follicles analyzed per treatment. The percentage of intact follicles decreased ($P < 0.05$) from Day 0 to Day 18 in all treatments, except for the control. Moreover, this percentage tended to decrease ($P < 0.08$) only in the A4 + SeqFSH treatment from Day 12 to Day 18 of culture (Fig. 1). Except for the control, the percentage of intact follicle decreased ($P < 0.05$) from Day 0 to Day 18 and tended to decrease ($P < 0.08$) only in the A4 + SeqFSH treatment from Day 12 to Day 18. In addition, this treatment was lower on Day 18 compared with the control ($P < 0.05$). The effect of treatments on follicle survival probability was evaluated by Kaplan–Meier method (Fig. 2). The only treatment that found a reduction (73.8%; $P < 0.05$) in follicle survival on Day 18 was the A4 + SeqFSH when compared with the control group.

The rate of degenerated follicles was similar ($P > 0.05$) among the treatments (Fig. 3). However, from Day 12 to Day 18 the A4 + SeqFSH treatment showed an increase ($P < 0.05$) in the percentage (11.9%) of degenerated follicles. The percentage of follicular extrusion is shown (Fig. 4). No difference was observed among treatments ($P > 0.05$). However, treatments containing FSH increased ($P < 0.05$) the percentage of extruded follicles from Day 0 to Day 18.

3.2. Follicular diameter and growth rate

The average values of follicular diameter during IVC of isolated preantral follicles are summarized (Table 1). A progressive increase ($P < 0.05$) in follicular diameter was observed in all treatments during the culture period.

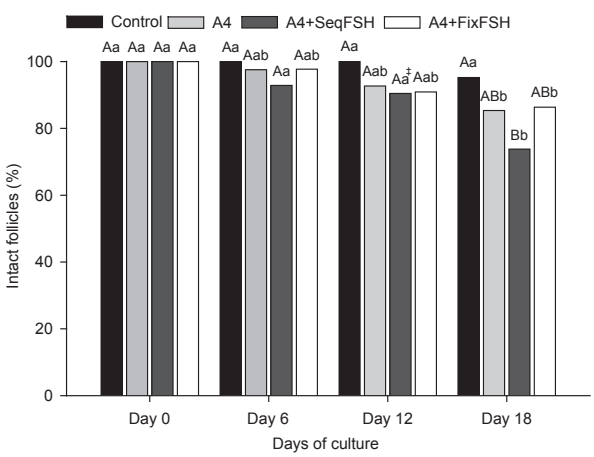

Fig. 1. Percentage of intact follicles during IVC in medium containing androstenedione associated or not with fixed and sequential concentrations of FSH. Control: α-MEM$^+$; A4: α-MEM$^+$ plus androstenedione; A4 + SeqFSH: α-MEM$^+$ plus androstenedione and sequential concentrations of rbFSH; A4 + FixFSH: α-MEM$^+$ plus androstenedione and fixed concentrations of rbFSH. $^{(A, B)}$Within day, uncommon uppercase letters indicate difference ($P < 0.05$). $^{(a, b)}$Within treatment, uncommon lowercase letters indicate difference ($P < 0.05$). ‡Tended to differ from Day 18 ($P < 0.08$).

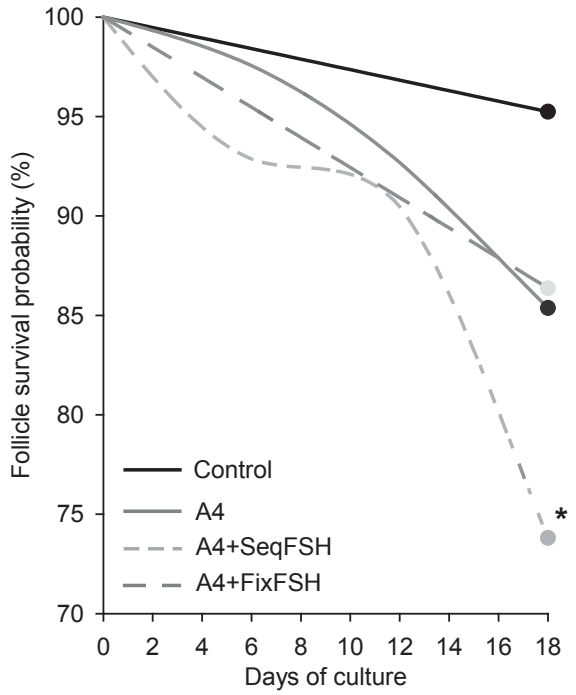

Fig. 2. Follicle survival probability (Kaplan–Meier plot) during IVC in medium containing androstenedione associated or not with fixed and sequential concentrations of FSH. Control: α-MEM$^+$; A4: α-MEM$^+$ plus androstenedione; A4 + SeqFSH: α-MEM$^+$ plus androstenedione and sequential concentrations of rbFSH; A4 + FixFSH: α-MEM$^+$ plus androstenedione and fixed concentrations of rbFSH. *Differed from control ($P < 0.05$).

However, the follicle diameter was similar ($P > 0.05$) among treatments irrespective of the culture period. The daily growth rate is summarized (Table 2), and the A4 + SeqFSH treatment tended ($P < 0.09$) to be higher than control group in the second-third of culture period (D6-D12). Moreover, only A4 + SeqFSH treatment the growth rate tended to be higher ($P < 0.07$) in the second-third compared with the first-third of culture. The frequency distributions of follicles classified according to growing rates is shown (Fig. 5A,B). We observed that the A4 + SeqFSH treatment presented a reduction ($P < 0.05$) in the percentage of slow-growing follicles compared with control and A4 treatments. In addition, FSH treatments showed a higher ($P > 0.05$) percentage of fast-growing follicles compared with control (Fig. 5B).

3.3. Antrum formation

Regardless of treatment, the percentage of antrum formation increased ($P < 0.05$) from Day 0 until Day 12 of culture and remained constant until the end of the culture period (Table 3). A positive correlation between antrum formation and follicular diameter during IVC was observed by logistic regression analysis (Fig. 6). Therefore, the mean growth rate of follicles that formed antrum followed by degeneration and extrusion was 1.8-fold superior than follicles that maintained the antrum (Fig. 7). When the

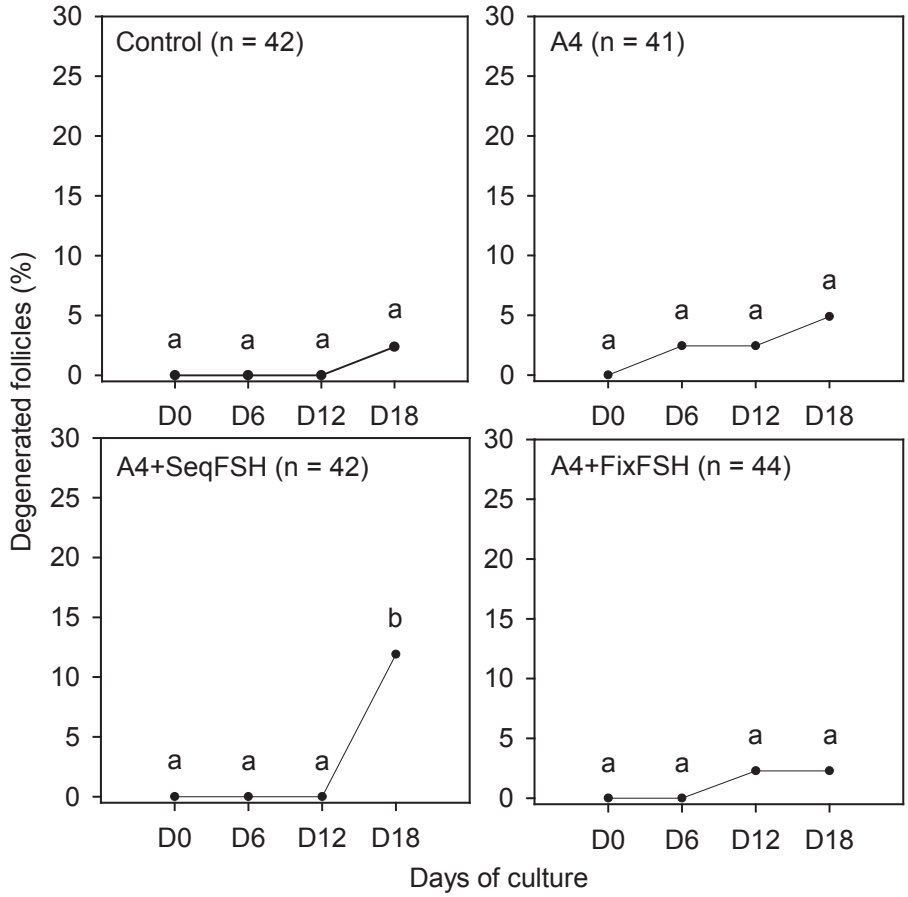

Fig. 3. Percentage of degenerated follicles during IVC in medium containing androstenedione associated or not with fixed and sequential concentrations of FSH. Control: α-MEM$^+$; A4: α-MEM$^+$ plus androstenedione; A4 + SeqFSH: α-MEM$^+$ plus androstenedione and sequential concentrations of rbFSH; A4 + FixFSH: α-MEM$^+$ plus androstenedione and fixed concentrations of rbFSH. $^{(a, b)}$Within treatment, uncommon lowercase letters indicate difference ($P < 0.05$). Among treatments within the same day ($P > 0.05$).

treatments were compared to each other using these end points (Fig. 8), the A4 + SeqFSH had a decrease ($P < 0.05$) in the percentage of follicles that maintained the antrum but an increase ($P < 0.05$) in the percentage of follicles that degenerated or extruded after antrum formation compared control group. These results could be confirmed by the positive association between antrum formation phenotypes and follicular growth rates (Fig. 9).

3.4. Oocyte parameters and chromatin configuration

No difference in the oocyte recovery rate (≥110 μm), oocyte diameter (μm), and oocyte viability (%) were observed among the treatments ($P > 0.05$) after 18 days of culture (data not shown). The A4 + SeqFSH treatment had a lower percentage of meiotic resumption ($P < 0.05$) than the control group and tended to differ ($P < 0.06$) from A4 + FixFSH treatment. Metaphase II was observed only in the control and A4 + FixFSH treatments (Fig. 10). Finally, as expected a positive relationship between chromatin configuration and oocyte diameter was observed using a regression analysis (Fig. 11).

3.5. Hormonal production

Regardless of treatment, androstenedione concentrations were similar ($P > 0.05$) between 6 and 18 days of culture. However, at Day 18 of culture, there was a decrease in androstenedione levels of A4 + SeqFSH compared with A4 (tended to differ; $P < 0.07$) and A4 + FixFSH treatments ($P < 0.05$; Fig. 12). No difference ($P > 0.05$) was observed among treatments on the estradiol concentration during the IVC (data not shown).

4. Discussion

In general, we observed that androstenedione, associated with increasing concentrations of FSH (A4 + SeqFSH) hampered the follicular survival and the ability of *in vitro* grown oocytes to resume meiosis.

Previous studies in caprines have shown that the presence of increasing concentrations of FSH and the addition of others substances such as activin A [22] EGF [23] and insulin-like growth factor-II [24] did not impair follicular development and improved meiosis resumption of the

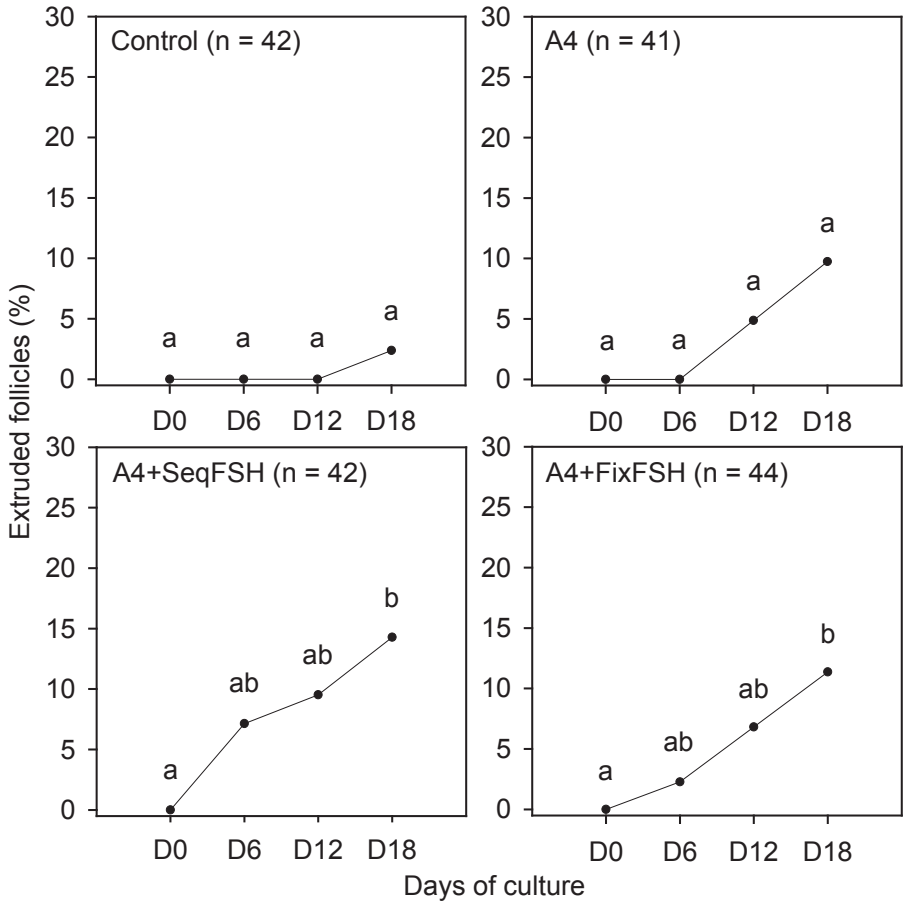

Fig. 4. Percentage of extruded follicles during IVC in medium containing androstenedione associated or not with fixed and sequential concentrations of FSH. Control: α-MEM$^+$; A4: α-MEM$^+$ plus androstenedione; A4 + SeqFSH: α-MEM$^+$ plus androstenedione and sequential concentrations of rbFSH; A4 + FixFSH: α-MEM$^+$ plus androstenedione and fixed concentrations of rbFSH. [a, b]Within treatment, uncommon lowercase letters indicate difference ($P < 0.05$). Among treatments within the same day ($P > 0.05$).

oocytes recovered from *in vitro* grown preantral follicles. Unlike the positive effects of those substances associated with increasing FSH concentrations, in our study, the androstenedione caused a deleterious effect on the IVC of preantral follicles, when associated with increasing concentrations of FSH and no effects when associated with fixed FSH concentration. Although we expected positive results, these findings clearly show the timeliness of this study.

In the present study, the percentage of morphologically intact follicles, and the follicular survival probability decreased, whereas the rate of degenerated follicles increased at the end of culture in the A4 + SeqFSH treatment. Several authors have described that during the IVC of preantral follicles, the addition of FSH either in increasing concentrations [3] or in decreasing concentrations associated to dihydrotestosterone [7] increased follicle survival. It is well known that follicular growth and atresia depends on

Table 1
Mean (±SEM) diameter of normal follicles cultured *in vitro* for 18 days in medium containing androstenedione associated or not with fixed and sequential concentrations of FSH.

Treatments	Follicular diameter (μm)			
	Day 0	Day 6	Day 12	Day 18
Control	202.1 ± 7.4[a]	238.7 ± 12.1[b]	258.2 ± 13.9[c]	289.3 ± 18.8[d]
A4	213.4 ± 7.5[a]	240.4 ± 11.1[b]	271.6 ± 14.0[c]	311.5 ± 21.3[d]
A4 + SeqFSH	202.1 ± 9.4[a]	224.8 ± 12.8[b]	278.9 ± 18.6[c]	317.9 ± 29.9[d]
A4 + FixFSH	208.5 ± 7.9[a]	240.9 ± 11.1[b]	293.3 ± 18.4[c]	325.9 ± 26.3[d]

Control: α-MEM$^+$; A4: α-MEM$^+$ plus androstenedione; A4 + SeqFSH: α-MEM$^+$ plus androstenedione and sequential concentrations of rbFSH; A4 + FixFSH: α-MEM$^+$ plus androstenedione and fixed concentrations of rbFSH.
[a, b, c, d]Within treatment, uncommon lowercase letters indicate difference ($P < 0.05$). Among treatments within the same day ($P > 0.05$).
Abbreviation: SEM, standard error of mean.

Table 2
Mean (±SEM) daily growth of normal follicles cultured *in vitro* for 18 days in medium containing androstenedione associated or not with fixed and sequential concentrations of FSH.

Treatments	Follicular growth (μm/day) on different intervals of culture			
	D0-D6	D6-D12	D12-D18	Overall (D0-D18)
Control	6.1 ± 1.4 ^{Aa}	3.2 ± 1.6 ^{Aa}	5.2 ± 1.5 ^{Aa}	4.8 ± 0.8^{A}
A4	4.5 ± 1.4 ^{Aa}	5.2 ± 2.0 ^{ABa}	6.6 ± 1.6 ^{Aa}	5.4 ± 1.1^{A}
A4 + SeqFSH	3.8 ± 2.0 ^{Aa}	9.0 ± 2.2^{Bab}	6.5 ± 2.7 ^{Aab}	6.4 ± 1.5^{A}
A4 + FixFSH	5.4 ± 1.4 ^{Aa}	8.7 ± 2.1 ^{ABa}	5.4 ± 1.8 ^{Aa}	6.5 ± 1.2^{A}

Control: α-MEM^{+}; A4: α-MEM^{+} plus androstenedione; A4 + SeqFSH: α-MEM^{+} plus androstenedione and sequential concentrations of rbFSH; A4 + FixFSH: α-MEM^{+} plus androstenedione and fixed concentrations of rbFSH.
$^{A, B}$Within interval time, uncommon uppercase letters indicate difference ($P < 0.05$).
Abbreviation: SEM, standard error of mean.
^{a} Tended to differ from control group within the same interval of culture ($P < 0.09$).$^{a, b}$Within treatment, uncommon lowercase letters indicate difference ($P < 0.05$).
^{b} Tended to differ from D0-D6 interval ($P < 0.07$).

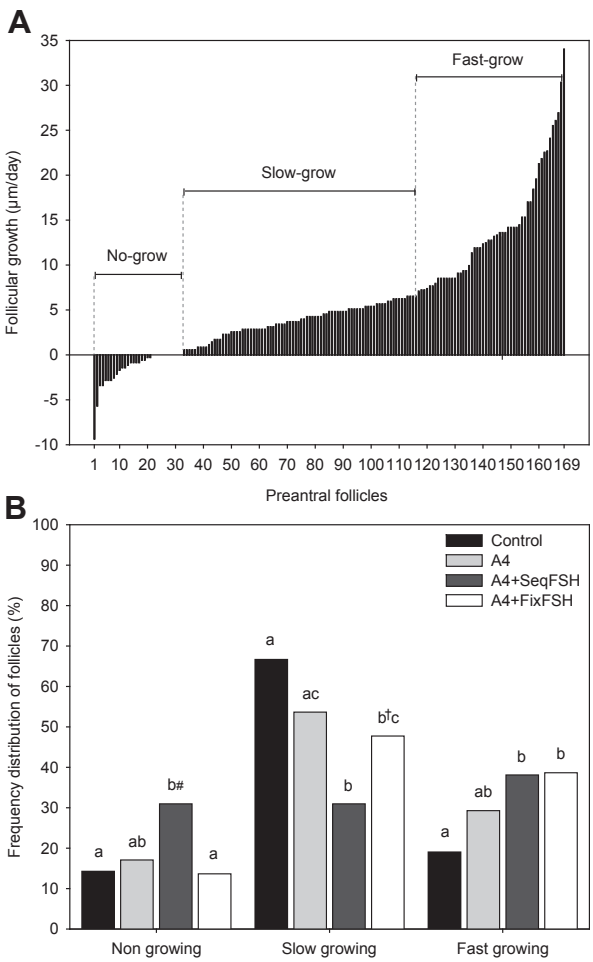

Fig. 5. (A) Representation of follicles (n = 169) classified according to growth rate in nongrowing, slow-, and fast-growing. (B) Frequency distributions of follicles in growing categories during IVC in medium containing androstenedione associated or not with fixed and sequential concentrations of FSH. Control: α-MEM^{+}; A4: α-MEM^{+} plus androstenedione; A4 + SeqFSH: α-MEM^{+} plus androstenedione and sequential concentrations of rbFSH; A4 + FixFSH: α-MEM^{+} plus androstenedione and fixed concentrations of rbFSH. $^{(a, b)}$Within the same growing categories, uncommon lowercase letters indicate difference ($P < 0.05$). $^{\#}$Tended to differ from control ($P < 0.06$). †Tended to differ from control ($P < 0.07$).

a delicate balance between gonadotropin and paracrine factors [25]. Therefore, we suggest that increasing concentrations of FSH associated with androstenedione may cause a negative effect, compromising the follicular physiology through the inappropriate proliferation of GC [26].

In relation to extrusion rates, FSH treatments (fixed and sequential) presented an increase of the rate of extruded follicles between Day 0 and Day18. Androgens increase the expression of FSH receptor (FSHR) in the GC [27,28]; therefore, we propose that the association of androstenedione with FSH may have potentiated the action of this hormone in the intrafollicular environment, hyperstimulating the proliferation of GC [26] and consequently, causing rupture of the basal membrane and releasing the cumulus-oocyte complexes. These findings are supported by the fact that FSH treatments presented a higher

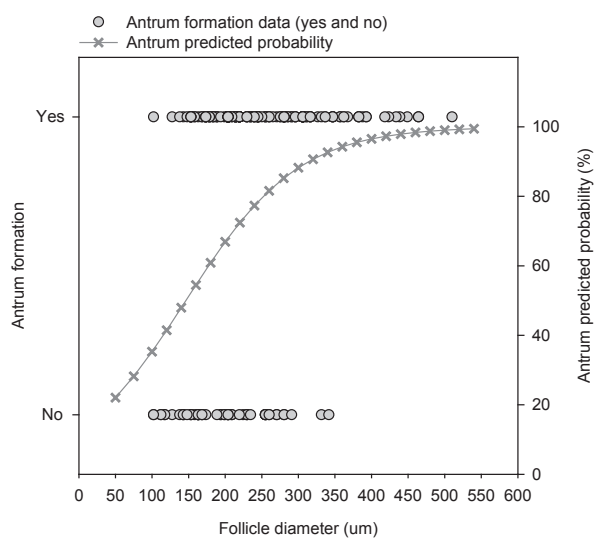

Fig. 6. Relationship between antrum formation and follicle diameter. Each point of the graph is a follicle recorded during IVC (n = 169). The follicles evaluated were defined by binary values (without antrum formation = 0; presence of antrum formation = 1) to dependent variable. A logistic regression (antrum predicted probability) is represented by the equation and the black line (Logit P = −1.916 + [0.0131 × follicle diameter]; $P < 0.05$).

Table 3
Percentage of antrum formation during IVC in medium containing androstenedione associated or not with fixed and sequential concentrations of FSH.

Treatments	Antrum formation (%)			
	Day 0	Day 6	Day 12	Day 18
Control	0.0 (0/42)[a]	66.6 (28/42)[b]	95.2 (40/42)[c]	95.2 (40/42)[c]
A4	0.0 (0/41)[a]	68.2 (28/41)[b]	90.2 (37/41)[c]	90.2 (37/41)[c]
A4 + SeqFSH	0.0 (0/42)[a]	64.2 (27/42)[b]	85.7 (36/42)[c]	85.7 (36/42)[c]
A4 + FixFSH	0.0 (0/44)[a]	65.9 (29/44)[b]	90.9 (40/44)[c]	90.9 (40/44)[c]

Control: α-MEM$^+$; A4: α-MEM$^+$ plus androstenedione; A4 + SeqFSH: α-MEM$^+$ plus androstenedione and sequential concentrations of rbFSH; A4 + FixFSH: α-MEM$^+$ plus androstenedione and fixed concentrations of rbFSH.
[a, b, c]Within treatment, uncommon lowercase letters indicate difference ($P < 0.05$). Among treatments within the same day ($P > 0.05$).

proportion of follicles in the fast-growing category than control group. In addition, using linear regression analysis it was possible to observe a correlation between fast-growing follicles and follicles that degenerated and/or extruded after antrum formation, implying that follicles require a slow growth rate to maintain follicular viability.

In the present study, the A4 + SeqFSH treatment was the only one that had lower percentage of follicles that formed antrum and remained intact until the end of the culture when compared with control. This can be explained by the high rates of degeneration and/or extrusion after antrum formation observed in this treatment. Other studies tested the effect of different concentrations of androstenedione (10 and 100 ng/mL) in medium that was FSH-free on the IVC of bovine early antral follicles and found that both concentrations were able to maintain the antrum intact until the end of culture [29].

This is the first study that evaluated the production of androstenedione during the IVC of goat preantral follicles. Despite the difference in androstenedione concentrations at the onset of culture (0 ng/mL-control vs. 10 ng/mL-A4 treatments), the androstenedione levels on Day 6 were equivalent between control and A4 treatments. In other words, the concentration of androstenedione was reduced at least 66 times in A4 treatments indicating a high rate of metabolization of androstenedione by cultured follicles in these treatments. In addition, follicles cultured in the medium containing low insulin were able to produce adequate levels of androstenedione, resulting in equivalent production of estradiol both on Day 6 and 18 of culture. This suggests that the excessive metabolization of androstenedione was not used expressly for estradiol production. During the steroidogenesis process, androstenedione can be converted either to: (1) testosterone by 17β-HSD (17β-hydroxysteroid dehydrogenase) which itself can then be aromatized into estradiol by P450arom (CYP19) in CG (A4→T→E2), (2) estrone by P450arom (CYP19) for subsequent conversion in estradiol by 17β-HSD in CG (A4→E1→E2), or (3) testosterone by 17β-HSD in TC which itself can then be converted into dihydrotestosterone by 5α-reductase in GC (A4→T→DHT) [8]. Therefore, in our study, we suggest that the major pathway used by the follicles cultured in A4 treatments was A4→DHT since the

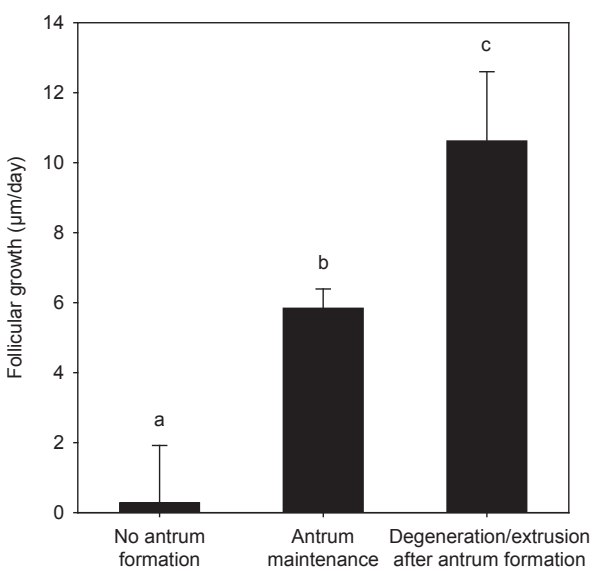

Fig. 7. Mean (±SEM) daily growth rate of follicles according to their ability to form antrum and maintain normal morphology. [a, b, c]Uncommon lowercase letters indicate difference ($P < 0.05$). SEM, standard error of mean.

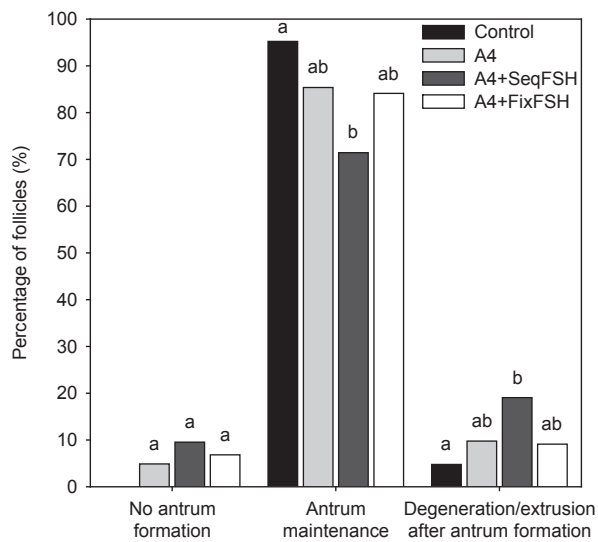

Fig. 8. Effect of the addition of androstenedione associated or not with fixed and sequential concentrations of FSH on the antrum formation and maintenance of normal morphology. Control: α-MEM$^+$; A4: α-MEM$^+$ plus androstenedione; A4 + SeqFSH: α-MEM$^+$ plus androstenedione and sequential concentrations of rbFSH; A4 + FixFSH: α-MEM$^+$ plus androstenedione and fixed concentrations of rbFSH. [a, b]Within the same antrum conditions, uncommon lowercase letters indicate difference ($P < 0.05$).

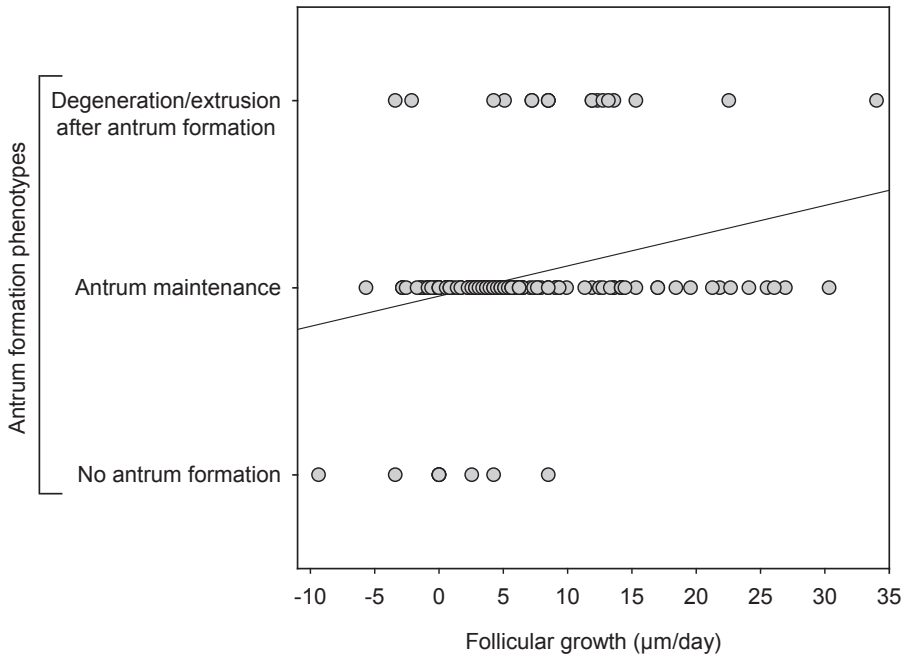

Fig. 9. Relationship between antrum phenotypes conditions and follicular growth. Each point of graph is a follicle recorded during IVC (n = 169). The follicles evaluated were defined by binary values (absence of antrum formation = 1; antrum maintenance = 2; follicles degenerated and/or extruded after antrum formation = 3) to dependent variable. A linear regression is represented by the equation and the black line (antrum formation conditions = 1.955 + [0.0162 × follicular growth]; $R^2 = 0.08$; r = 0.28; $P < 0.001$).

estradiol production did not differ among treatments regardless the culture period and the testosterone production was below the detection limit. This assumption can be supported by Bogovish and Richards [30] which found the ability of GC from rodents to generate dihydrotestosterone when treated with androstenedione. Moreover, Wu et al. [31] reported that the addition of testosterone in medium containing FSH or not promoted a

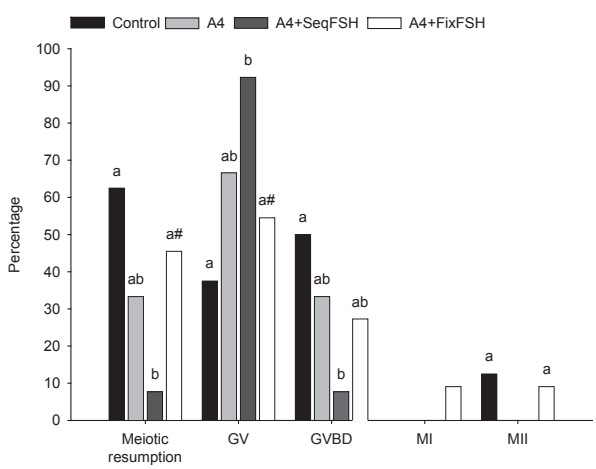

Fig. 10. Percentage of meiotic resumption, germinal vesicle, germinal vesicle breakdown, metaphase I, and metaphase II of oocytes from preantral follicles cultured *in vitro* in medium containing androstenedione associated or not with fixed and sequential concentrations of FSH. Control: α-MEM$^+$; A4: α-MEM$^+$ plus androstenedione; A4 + SeqFSH: α-MEM$^+$ plus androstenedione and sequential concentrations of rbFSH; A4 + FixFSH: α-MEM$^+$ plus androstenedione and fixed concentrations of rbFSH. $^{(a, b)}$Within the same parameter, values with different letters differ ($P < 0.05$). $^{\#}$Tended to differ from A4 + SeqFSH ($P < 0.06$).

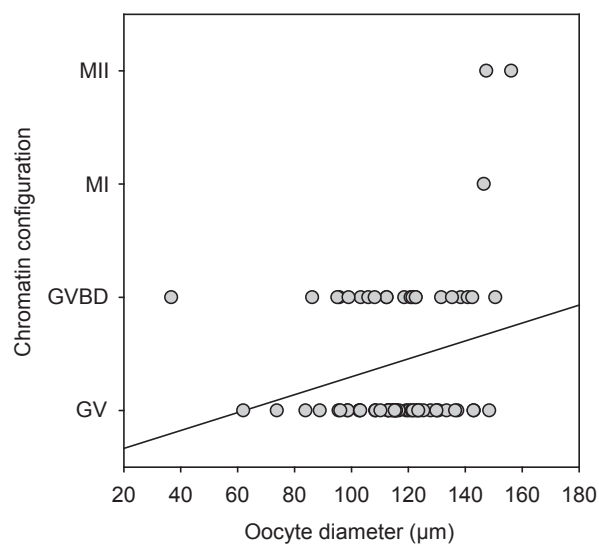

Fig. 11. Regression analysis of the chromatin configuration (GV, GVBD, MI, and MII) and oocyte diameter. Each point of graph is an oocyte evaluated after IVM (n = 68). The oocytes evaluated were defined by values (GV = 2; GVBD = 3; MI = 4; and MII = 5) to chromatin configuration (dependent variable). A linear regression is represented by the equation and the black line (chromatin configuration = 1.507 + [0.00791 × oocyte diameter]; $R^2 = 0.06$; r = 0.24; $P < 0.05$). GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II.

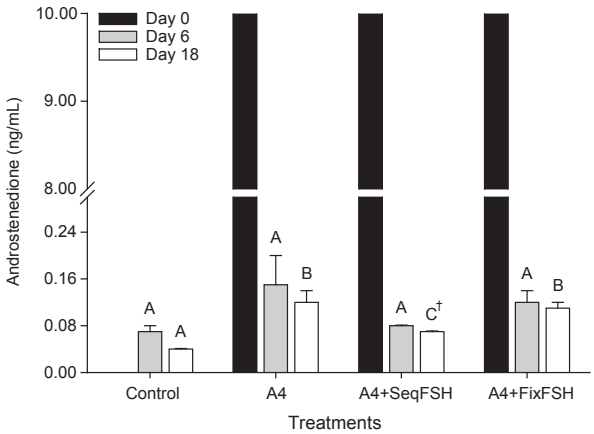

Fig. 12. Mean (±SEM) androstenedione levels from preantral follicles cultured *in vitro* in medium containing androstenedione associated or not with fixed and sequential concentrations of FSH. Control: α-MEM$^+$; A4: α-MEM$^+$ plus androstenedione; A4 + SeqFSH: α-MEM$^+$ plus androstenedione and sequential concentrations of rbFSH; A4 + FixFSH: α-MEM$^+$ plus androstenedione and fixed concentrations of rbFSH. $^{(A, B, C)}$Within day, uncommon uppercase letters indicate difference ($P < 0.05$). †Tended to differ from A4 ($P < 0.07$). Within treatment between days ($P > 0.05$). SEM, standard error of mean.

stimulatory effect of CYP19 in mice GC, which correlated with estradiol production.

The A4 + SeqFSH treatment reported lower rates of meiosis resumption than control after oocyte IVM. The control medium used in our experiment was previously developed by our team [20] which defined the appropriated insulin concentration as 10 ng/mL. Insulin is a major factor related to follicular survival, growth, and production of steroids hormones [20]. Like androgens [27], studies have reported that insulin increases the expression of FSHR [32,33]. We believe that androstenedione associated with insulin enhanced the action of FSH on the IVC of preantral follicles. The high FSH concentrations used in the A4 + SeqFSH treatment caused a deleterious effect on meiosis resumption. This result is in agreement with Sánchez et al. [26] that reported that higher concentrations of FSH increased the levels of LH receptors in mice cumulus cells, compromising oocyte competence, since the upregulation of LH receptors is related to low-oocyte quality [34].

In our study, surviving follicles were divided into 3 categories (nongrowing, slow- and fast-growing follicles), according to their growth rates. The growth rates are an important parameter to evaluate the efficiency of follicular development in culture. However, in our study, accelerated follicle growth was correlated with degeneration and/or extrusion which may allow it to be used as a tool to predict follicular fate *in vitro*. On the other hand, Xu et al. [35] reported that in the culture of monkeys preantral follicles, the production of anti-Mullerian hormone was correlated with follicular growth, wherein early anti-Mullerian hormone production can predict further development of cultured preantral follicles. Other studies using activin A in the culture medium of isolated caprine preantral follicles found that the treatment with the highest percentage of fast-growing follicles also presented high meiotic resumption rates [23]. Differences in the species, in the medium composition and in the classification on the follicle growth categories may explain this controversial finding. For instance, in the culture of isolated caprine preantral follicles, Silva et al. [23] classified fast-growing follicles as those which showed growth rate higher than 10 μm/day, whereas in our study the follicles that had a daily growth between 7.1 and 34 μm/day were classified as fast-growing follicles. Besides the presence of androstenedione in the majority of our treatments, Silva et al. [23] used higher insulin concentration in the basic medium. This indicates that in the presence of androstenedione and increasing concentrations of FSH the acceleration of follicular development was detrimental for efficiency of caprine preantral follicles cultured *in vitro*.

In summary, the addition of androstenedione alone or associated with fixed concentrations of FSH did not improve either follicular survival, development or steroidal levels after IVC. Moreover, increasing FSH concentrations associated with androstenedione had a detrimental effect on follicle survival and oocyte meiotic resumption. Finally, this study found that accelerated follicle growth negatively affected the morphology of caprine preantral follicle cultured *in vitro*.

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Competing Interests

None of the authors declared having any conflicts of interest.

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