Interaction between ascorbic acid and follicle-stimulating hormone maintains follicular viability after long-term in vitro culture of caprine preantral follicles

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Abstract

This study evaluates the effects of ascorbic acid and its interaction with follicle-stimulating hormone (FSH) on the morphology, activation, and in vitro growth of caprine preantral follicles. Ovarian fragments were cultured for 1, 7, or 14 d in minimum essential medium (MEM) containing ascorbic acid (50 or 100 μg/mL), FSH (50 ng/mL), or both of these substances. Ovarian tissue that was either fresh (control) or cultured for 1, 7, or 14 d was processed for histological and ultrastructural evaluation. The results showed that after 14 d of culture, medium supplemented with 50 μg/mL of ascorbic acid alone or combined with FSH showed higher rates of follicular survival compared with MEM. After 7 d of culture, FSH, ascorbic acid at 50 μg/mL with or without FSH, and ascorbic acid at 100 μg/mL increased the percentage of follicular activation compared to fresh control. In addition, FSH alone significantly increased the percentage of growing follicles after 14 d. The combination of 50 μg/mL of ascorbic acid and FSH promoted a significant increase in oocyte and follicular diameter after 7 d of culture. Ultrastructural and fluorescent analysis confirmed the integrity of follicles cultured with 50 μg/mL of ascorbic acid and FSH after 14 d. In conclusion, the combination of 50 μg/mL of ascorbic acid and FSH maintained follicular integrity and promoted follicular activation and growth after long-term in vitro culture of caprine preantral follicles.

Keywords: Ascorbic acid; FSH; Preantral; Caprine; In vitro culture

1. Introduction

The development of modern reproductive biotechnologies allows a better understanding of ovarian and embryonic physiology, with the goal of genetic improvement and increased productivity of high-economic-value animals and endangered species. Nevertheless, the large-scale use of these procedures depends on the availability of mature oocytes, which are present in only a small number of follicles in the ovary. Since 99.9% of the

follicles present in the ovary are eliminated by atresia [1], and 90% of the ovarian follicular population consists of primordial follicles, several studies have been done to develop a culture system that allows in vitro growth and maturation of oocytes from preantral follicles [2]. It is known that in larger species, early follicular development follows a very lengthy and complex process [3]. Therefore, an extended culture period may be required for preantral follicle development. However, few long-term in vitro culture studies achieved follicular activation, namely, the transition from primordial to growing follicles in ovines [4] and humans [5] and [6].

Among the protective substances that can be added to the culture medium of preantral follicles, it is important to mention ascorbic acid. At physiological concentrations, ascorbic acid, or vitamin C, is a potent free radical scavenger, protecting cells against the damage caused by reactive oxygen species (ROS) [7]. The antioxidant properties of ascorbic acid are attributed to its capacity to significantly reduce the damage from ROS, forming ascorbate as a stable free radical [8]. This mechanism performs a large number of cytoprotective functions under physiological conditions, including the prevention of DNA mutations induced by oxidation [9] and [10], protection against lipidic peroxidation [11] and [12], and repair of oxidized amino acids for maintenance of protein integrity [11] and [13]. Ascorbic acid accumulates in granulosa cells, the inner theca, luteal cells, and oocytes [14]. In addition, during follicular growth, ovulation, and corpus luteum formation, the basement membrane and extracellular matrix undergo constant remodeling, thus requiring a great deal of collagen, and ascorbic acid plays a role in the process of collagen synthesis [15]. Moreover, it was demonstrated that this antioxidant is able to reduce apoptosis in follicles from cows [14] and rats [16].

Another substance that is commonly used for in vitro culture of preantral follicles is the follicle-stimulating hormone (FSH). Follicle-stimulating hormone receptors are expressed in granulosa cells [17] and [18] from the primary follicle stage onward [19]. However, although these receptors are not present in primordial follicles, FSH seems to play an indirect role in early follicular development through the release of paracrine factors produced by large follicles or ovarian stroma cells [20]. Recently, after culture of caprine ovarian tissue in the presence of 50 ng/mL of FSH, Matos et al. [21], showed the maintenance of ultrastructural integrity and follicular growth for only 7 d of culture. However, after this period, the medium used was not able to preserve satisfactory follicular morphology. Several studies have demonstrated that FSH can inhibit apoptosis in preantral follicles cultured in vitro in humans [22], mice [23], and rats [24].

Despite the importance of FSH and ascorbic acid in the in vitro culture studies, there are no reports about the interaction of ascorbic acid and FSH on caprine preantral follicles

cultured in vitro. In this study, we evaluated the effects of ascorbic acid in conjunction with FSH on the viability, activation, and in vitro growth of caprine preantral follicles after long-term culture.

2. Material and methods

2.1. Chemicals

Unless stated otherwise, the culture media, ascorbic acid, and other chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Source of ovaries

Ovarian cortical tissues (n = 10 ovaries) were collected at a local slaughterhouse from 5 adult (1-3 years old), mixed-breed goats (Capra hircus). Immediately postmortem, the ovaries were washed in 70% alcohol for 10 seconds followed by 2 washes with minimum essential medium (MEM) supplemented with 100 µg/mL penicillin and 100 µg/mL streptomycin. The pairs of ovaries were transported, in MEM at 4 °C, within 1 h to the laboratory.

2.3. Experimental protocol

Our organ culture system was described in detail previously [21]. Ovarian tissue samples from each ovarian pair were cut into 19 slices (9 mm3) using a needle and scalpel under sterile conditions. The tissue pieces were then either directly fixed for histological and ultrastructural analysis (fresh control) or placed in culture for 1, 7, or 14 d. Caprine tissues were transferred to 24-well culture dishes containing 1 mL of culture medium. The culture was performed at 39 °C in 5% CO2 in a humidified incubator, and all the media were incubated for 1 h prior to use. The basic culture medium (control medium) consisted of MEM (pH 7.2-7.4) supplemented with insulin 6.25 ng/mL, transferrin 6.25 ng/mL and selenium 6.25 ng/mL (ITS), 0.23 mM pyruvate, 2 mM glutamine, 2 mM hypoxanthine, and 1.25 mg/mL bovine serum albumin (BSA), and this medium was called MEM+. For the experimental conditions, the medium was supplemented with ascorbic acid at 2 concentrations (50 or 100 µg/mL), FSH (50 ng/mL) (porcine FSH, Stimufol, FSH:LH = 20:1, donated by Dr. J.F. Beckers, Liège, Belgium), or the combinations of concentrations of ascorbic acid and FSH shown in Table 1. Each treatment

was repeated 5 times, and the culture medium was replenished every other day. The concentrations of ascorbic acid and FSH used in this work were chosen based on previous studies [14] and [21].

Table 1
Different media tested for the in vitro culture of caprine preantral follicles.

Culture medium	Abbreviation
MEM^+	MEM^+
MEM^++FSH (50 ng/mL)	FSH
MEM^++Ascorbic Acid (50 μg/mL)	AA50
MEM^++FSH+Ascorbic Acid (50 μg/mL)	FSH+AA50
MEM^++Ascorbic Acid (100 μg/mL)	AA100
MEM^++FSH+Ascorbic Acid (100 μg/mL)	FSH+AA100

2.4. Morphological analysis and assessment of in vitro follicular growth

Before culture (fresh control) and after 1, 7, or 14 d in culture, all of the pieces were fixed in Carnoy's solution for 12 h and then dehydrated in increasing concentrations of ethanol. After paraffin embedding (Synth, São Paulo, Brazil), the caprine tissue pieces were cut into 7-μm sections, and each section was mounted on a glass slide and stained using periodic acid Schiff - hematoxylin. Follicle stage and survival were assessed microscopically on serial sections. Slides were coded for unbiased analysis and examined by microscopy (Nikon, Japan) under 400X magnification.

The developmental stages of follicles have been defined previously [25] as primordial (1 layer of flattened granulosa cells around the oocyte) or growing follicles (intermediate: 1 layer of flattened to cuboidal granulosa cells; primary: 1 layer of cuboidal granulosa cells; and secondary: 2 or more layers of cuboidal granulosa cells around the oocyte). These follicles were classified individually as histologically normal when an intact oocyte was present, surrounded by granulosa cells that were well organized in 1 or more layers and that had no pycnotic nuclei. Atretic follicles were defined as those with a retracted oocyte, pycnotic nucleus, and/or disorganized granulosa cells detached from the basement membrane. Overall, 150 follicles were evaluated for each treatment (30 follicles per treatment in 1 repetition x 5 repetitions = 150 follicles).

To evaluate follicular activation, the percentages of healthy primordial and growing follicles were calculated before (fresh control) and after culture in each medium. In addition,

follicle and oocyte diameters were measured only in healthy follicles. Follicle diameter was recorded from edge to edge of the granulosa cell membrane or from the outside edge of the theca cell layer when present, and oocyte diameter was recorded from edge to edge of the oocyte membrane, both using a DS Cooled Camera Head DS-Ri1 coupled to a Nikon Eclipse 80i microscope and analyzed by Nikon NIS-Elements software (Nikon NIS-Elements 3.0, 2008). Two perpendicular diameters were recorded for each, and the average of these 2 values was reported as follicle diameter and oocyte diameter, respectively. Care was taken to count each follicle only once, as we have also done in our earlier studies [21] and [26]. Each follicle was examined in every section in which it appeared and matched with the same follicle on adjacent sections to avoid double-counting, thus ensuring that each follicle was counted only once, regardless of its size.

2.5. Ultrastructural analysis

Small pieces (1 mm3) of caprine ovarian tissue were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 4 h at room temperature. After fixation, fragments were post-fixed in 1% osmium tetroxide, 0.8% potassium ferricyanide, and 5 mM calcium chloride in 0.1 M sodium cacodylate buffer for 1 h. Subsequently, the samples were dehydrated through a gradient of acetone solutions and the tissues were embedded in Spurr. Semi-thin sections (3 μm) were cut on an ultramicrotome (Reichert Supernova, Heidelberg, Germany) and stained with toluidine blue for light microscopy studies. The ultrathin sections (60–70 nm) were contrasted with uranyl acetate and lead citrate, and examined under a Jeol 1011 (Jeol, Tokyo, Japan) transmission electron microscope. Parameters such as the density and integrity of ooplasmic and granulosa cell organelles, vacuolization, and basement membrane integrity were evaluated.

2.6. Assessment of preantral follicle viability by fluorescence

A viability study using an experimental protocol similar to that described in the above morphological investigation was performed with the objective of analyzing the effects of caprine ovarian tissue culture on the viability of preantral follicles. Based on the results of morphological and ultrastructural analysis, viability of follicles cultured with the concentration of ascorbic acid with or without FSH that provided the best outcome was further analyzed using a more accurate method of assessment based on fluorescent probes. Additional pairs of

ovaries (n = 2) were cut into fragments, from which 1 was immediately processed for follicle isolation.

Goat preantral follicles were isolated from ovarian fragments using the mechanical method described by Figueiredo et al. [27]. Briefly, with a tissue chopper (The Mickle Laboratory Engineering Co., Gomshal, Surrey, UK) adjusted to a sectioning interval of 75 µm, samples were cut into small pieces, which were placed in MEM, resuspended 40 times using a large Pasteur pipette (diameter of about 1600 µm), and subsequently resuspended 40 times with a smaller Pasteur pipette (diameter of approximately 600 µm) to dissociate preantral follicles from stroma. The material that was obtained was passed through 100-µm nylon mesh filters, resulting in a suspension containing preantral follicles smaller than 100 µm in diameter. This procedure was carried out within 10 min at room temperature.

Preantral follicles were analyzed using a 2-color fluorescence cell viability assay based on the simultaneous determination of live and dead cells by calcein-AM and ethidium homodimer-1, respectively. Although the first probe detected intracellular esterase activity of viable cells, the latter labeled nucleic acids of nonviable cells with plasma membrane disruption. The test was performed by adding 4 µM calcein-AM and 2 µM ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany) to the suspension of isolated follicles, followed by incubation at 37 °C for 15 min. After labeling, follicles were washed once by centrifugation at 100 × g for 5 min and resuspended in MEM, mounted on a glass microscope slide in 5 µL antifading medium (DABCO, Sigma, Deisenhofen, Germany) to prevent photobleaching, and finally examined using an a DMLB fluorescence microscope (Leica, Germany). The emitted fluorescent signals of calcein-AM and ethidium homodimer were collected at 488 and 568 nm, respectively. Oocytes and granulosa cells were considered live if the cytoplasm was stained positively with calcein-AM (green) and if chromatin was not labeled with ethidium homodimer (red).

2.7. Statistical analysis

Data were initially submitted to Kolmorogov-Smirnov and Bartlett tests to confirm normal distribution and homocedasticity, respectively. Two-way analysis of variance (ANOVA) was then carried out using general linear model procedures (SAS version 8.0, Inc., Cary, NC, 1996). The model used in ANOVA included medium, days of culture, and replicate as sources of variation. Dunnett's test was applied for comparison of control group against each treatment tested, and the Student Newman Keuls (SNK) test was used to compare percentages of

surviving primordial or developing follicles among treatments and days of culture. Differences among groups were considered significant when $P < 0.05$, and results were expressed as mean ± standard deviation (SD).

3. Results

3.1. Caprine preantral follicle survival after in vitro culture

A total of 2850 preantral follicles were analyzed by classical histology. Fig. 1A and C show normal follicles from fresh control and after culture in 50 µg/mL of ascorbic acid plus FSH, whereas Fig. 1B and D illustrate degenerated follicles after culture in MEM alone or with 50 µg/mL of ascorbic acid plus FSH, all after 14 d of culture. In the degenerated follicles, retracted oocytes, pycnotic nuclei, and disorganized granulosa cells were observed.

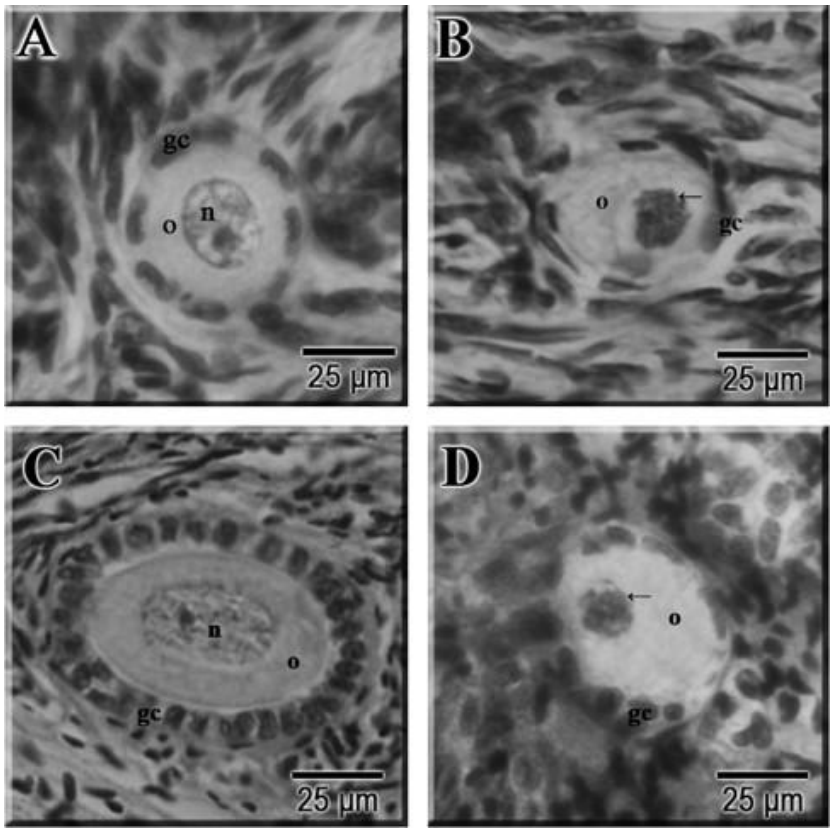


Fig. 1. Histological section (400X) of normal preantral follicles in fresh-control (A) or cultured for 14 d with follicle-stimulating hormone plus ascorbic acid (50 µg/mL) (C), and degenerated preantral follicles cultured for 14 d with minimum essential medium alone (B) or follicle-stimulating hormone plus ascorbic acid (50 µg/mL) (D), showing n–nucleus, gc- granulosa cell, o - oocyte. Note pycnotic nucleus (arrow).

The percentage of histologically normal preantral follicles in fresh controls and after 1, 7, or 14 d of in vitro culture is shown in Fig. 2. After 1, 7, or 14 d of culture, there was a significant reduction ($P < 0.05$) in the percentage of morphologically normal follicles in all treatments compared with fresh control, except when FSH was used in association with 50 µg/mL of ascorbic acid after 1 d of culture ($P > 0.05$). When all other treatments were compared with the culture performed with MEM+ alone, the addition of 50 µg/mL of ascorbic acid, 50 ng/mL of FSH, or both of these substances in the indicated concentrations significantly ($P < 0.05$) increased the percentage of normal follicles after 14 d. When the comparisons were done among the treatments on each day of culture, after 7 or 14 d, the combination of ascorbic acid (50 µg/mL) and FSH significantly increased the percentage of normal follicles compared with the combination of ascorbic acid (100 µg/mL) with FSH ($P < 0.05$). Medium supplemented with FSH alone maintained the percentage of normal follicles throughout the culture, that is, from day 1 to 7, and extended to d 14 of culture ($P > 0.05$). Nevertheless, there was a significant reduction in the percentage of follicular survival in the culture from d 1 to d 7 and out to d 14 after culture in MEM+ medium ($P < 0.05$). However, the addition of 50 µg/mL of ascorbic acid or the combination of 50 and 100 µg/mL of this antioxidant with FSH maintained the percentage of follicular survival between d 7 and d 14 of culture ($P > 0.05$).

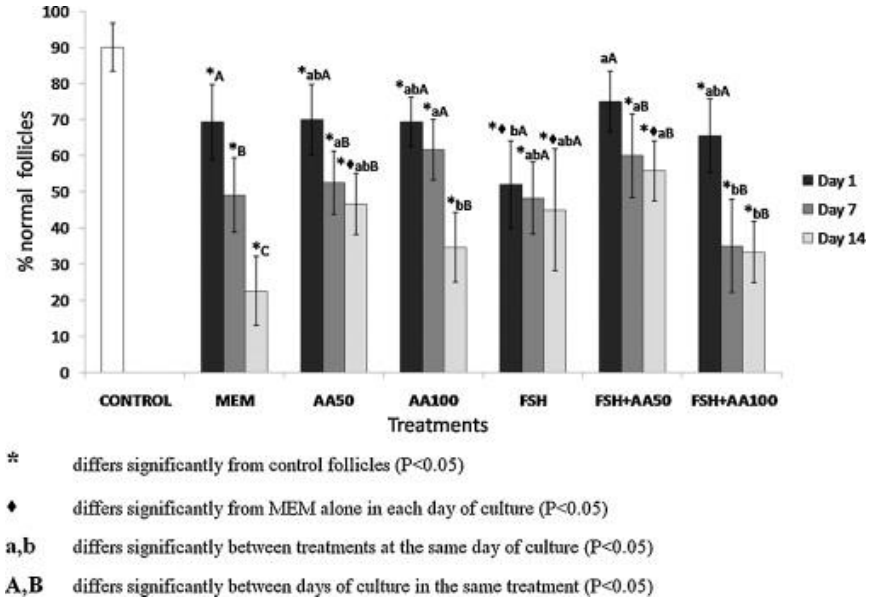


Fig. 2. Percentage of healthy preantral follicles in control (fresh tissue) and after 1, 7, or 14 d of culture in medium containing ascorbic acid and/or follicle-stimulating hormone.

3.2. Activation of caprine primordial follicles after in vitro culture

After 7 d of culture, there was a significant increase in the percentage of growing follicles in medium supplemented with 50 µg/mL of ascorbic acid and FSH alone or together, and in 100 µg/mL of ascorbic acid with FSH (Fig. 3) ($P < 0.05$). After 14 d, all treatments significantly increased the percentage of growing follicles compared to the fresh control ($P < 0.05$), except when the culture was performed in medium containing 100 µg/mL of ascorbic acid alone or 50 µg/mL of ascorbic acid with FSH ($P > 0.05$). With regard to the growing follicles, there was no significant difference among the treatments and MEM+ or among the treatments on the same day of culture ($P > 0.05$). In addition, when compared with d 1, only medium supplemented with FSH promoted an increase in the percentage of growing follicles after 14 d of culture ($P < 0.05$).

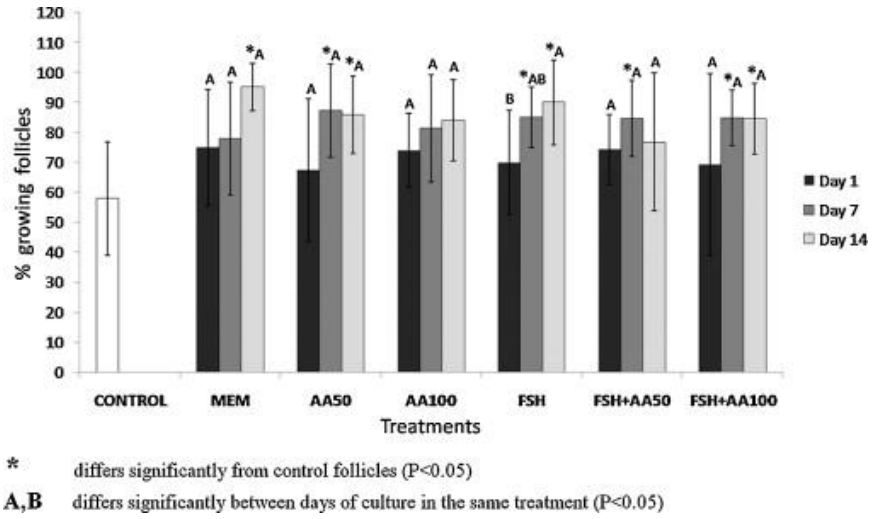
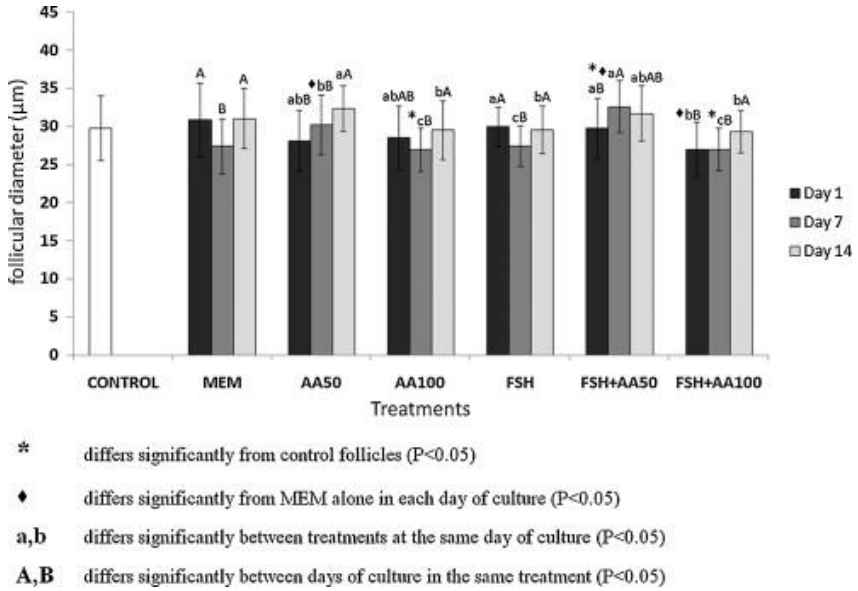


Fig. 3. Percentage of growing preantral follicles in control (fresh tissue) and after 1, 7. or 14 d of culture in medium containing ascorbic acid and/or follicle-stimulating hormone.

3.3. In vitro growth of caprine preantral follicles

After 7 d of culture, only medium supplemented with 50 µg/mL of ascorbic acid combined with FSH promoted a higher follicular diameter than fresh control (Fig. 4; $P < 0.05$). However, in this same period, treatments with 100 µg/mL of ascorbic acid alone or in combination with FSH significantly decreased follicular diameter compared with fresh control ($P < 0.05$). In addition, there was a significant increase in follicular diameter only when ascorbic acid was used at 50 µg/mL alone or with FSH compared with MEM+ ($P < 0.05$). When all treatments were compared to each other in the same culture period, only 50 µg/mL of ascorbic acid with FSH significantly increased follicular diameter after 7 d ($P < 0.05$). Moreover, after 14 d of culture, ascorbic acid at 50 µg/mL significantly increased follicular diameter,

compared with 100 µg/mL of ascorbic acid, FSH, or the combination of 100 µg/mL of ascorbic acid with FSH ($P < 0.05$). From d 7 to d 14 of the culture, all treatments significantly increased follicular diameter ($P < 0.05$), except when the medium was supplemented with 50 µg/mL of ascorbic acid with FSH, where a significant increase was observed from d 1 to d 7 ($P < 0.05$).



* differs significantly from control follicles ($P<0.05$)

♦ differs significantly from MEM alone in each day of culture ($P<0.05$)

a,b differs significantly between treatments at the same day of culture ($P<0.05$)

A,B differs significantly between days of culture in the same treatment ($P<0.05$)

Fig. 4. Mean follicular diameter (µm) in the control (fresh tissue) and after 1, 7, or 14 d of culture in medium containing ascorbic acid and/or follicle-stimulating hormone.

The use of 50 µg/mL of ascorbic acid with FSH or the antioxidant alone in the same concentration promoted a higher oocyte diameter than fresh control after 7 and 14 d of culture, respectively (Fig. 5; $P < 0.05$). After 7 d, only 50 µg/mL of ascorbic acid alone or combined with FSH significantly increased oocyte diameter compared with MEM+. However, after 1 d of culture, addition of 100 µg/mL of ascorbic acid with FSH promoted a significant reduction in oocyte diameter ($P < 0.05$) compared with MEM+. When the comparisons were done among all treatments after 7 d, medium containing 50 µg/mL of ascorbic acid alone or combined with FSH promoted a significant increase in oocyte diameter ($P < 0.05$). The same result was observed after 14 d with medium supplemented with 50 µg/mL of ascorbic acid alone, compared with FSH or the combination of ascorbic acid (100 µg/mL) and FSH ($P < 0.05$). As the culture progressed from 7 to 14 d, there was a significant increase in oocyte diameter in culture performed with 100 µg/mL of ascorbic acid alone or with FSH and with FSH alone ($P < 0.05$). Nevertheless, when the follicles were cultured with 50 µg/mL of ascorbic acid alone or with FSH, oocyte diameter increased throughout of the culture (from d 1 to 7) ($P < 0.05$).

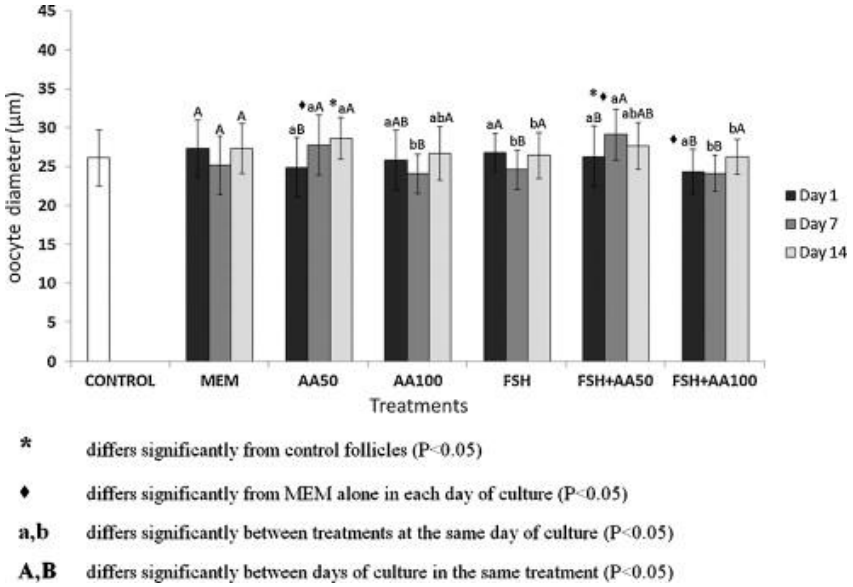


Fig. 5. Mean oocyte diameter (µm) in the control (fresh tissue) and after 1, 7, or 14 d of culture in medium containing ascorbic acid and/or follicle-stimulating hormone.

3.4. Ultrastructural analysis of caprine preantral follicles

For a better evaluation of follicular integrity, ultrastructural analysis was performed with tissues from fresh control as well as in those cultured for 14 d with 50 µg/mL of ascorbic acid or FSH, or 50 µg/mL of ascorbic acid and FSH, all of which showed satisfactory results at histological evaluation with regard to follicular health, growth, and activation. Ultrastructural features of follicles evaluated in fresh control (Fig. 6A) or in those cultured for 14 d with 50 µg/mL of ascorbic acid combined with FSH were similar and showed intact oocyte and nuclear membranes and large oocyte nuclei (Fig. 6B). There were organelles uniformly distributed in the ooplasm, especially mitochondria and endoplasmic reticulum. In addition, granulosa cells were ultrastructurally normal, showing an elongated and large nucleus with an irregular membrane and a high proportion of nucleus to cytoplasm. Nevertheless, follicles cultured with 50 µg/mL of ascorbic acid (Fig. 6C) or FSH alone showed signs of degeneration, such as high levels of cytoplasmic vacuolization, absence of integrity in the basal membrane, and disorganization of granulosa cells.

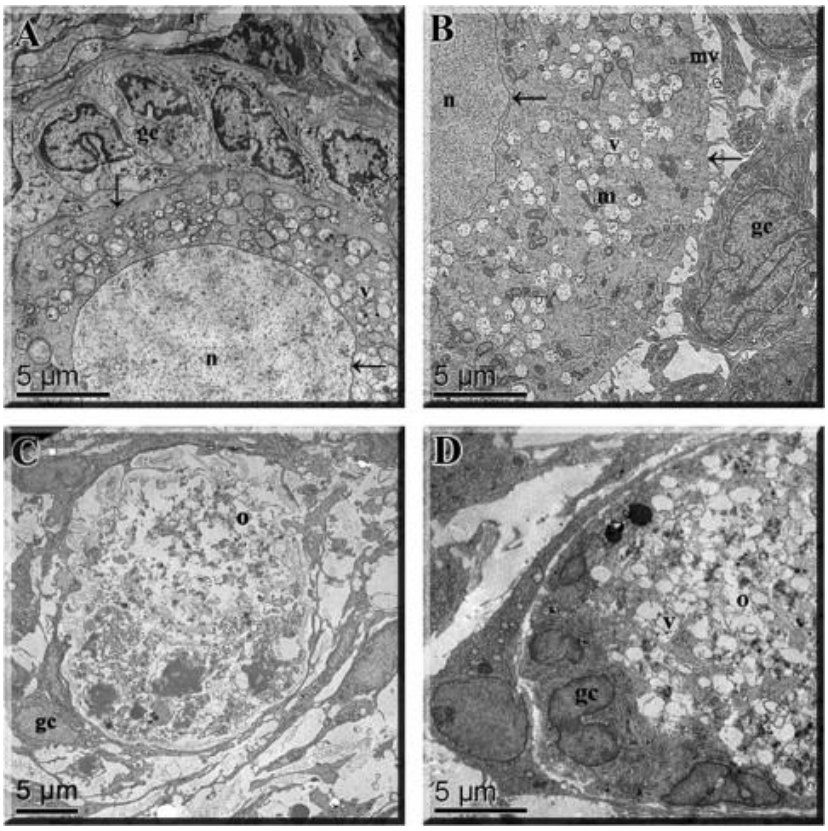


Fig. 6. Ultrastructural analysis of normal preantral intermediary follicles from fresh control (6000X) (A) and cultured for 14 d in medium containing follicle-stimulating hormone plus ascorbic acid (50 µg/mL) (6000X) (B). Degenerated intermediary follicle cultured in the presence of ascorbic acid (50 µg/mL) alone (5000X) (C) or minimum essential medium+ (MEM +) alone (6000X) (D), showing v–vacuoles, o–oocyte, n–nucleus, gc- granulosa cell, m- mitochondria, mv–microvillus, arrows- nuclear and oocyte membranes. Note in A and B: intact oocytes and nuclear membranes, nuclei with descondensed chromatin, granulosa cells with elongated nuclei, and a high proportion of nucleus to cytoplasm.

3.5. Viability assessment of follicles after culture

Based on the results of morphological and ultrastructural evaluation, goat preantral follicles were isolated from fresh control and from ovarian fragments cultured for 14 d with 50 µg/mL of ascorbic acid alone or in combination with FSH, and a viability trial using these treatments was performed. A fluorescence cell viability assay based on labeling of live and dead cells by calcein-AM and ethidium homodimer-1, respectively, was employed. The ultrastructural results were confirmed by fluorescent analysis, which showed viable follicles with oocyte and granulosa cells stained in green with calcein-AM in the fresh control (Fig. 7A-B), in preantral follicles cultured in 50 µg/mL of ascorbic acid with FSH (Fig. 7C-D), or with FSH alone (Fig. 7E-F). In contrast, degenerated follicles were stained in red with ethidium homodimer after 14 d of culture with 50 µg/mL of ascorbic acid alone (Fig. 7G-H).

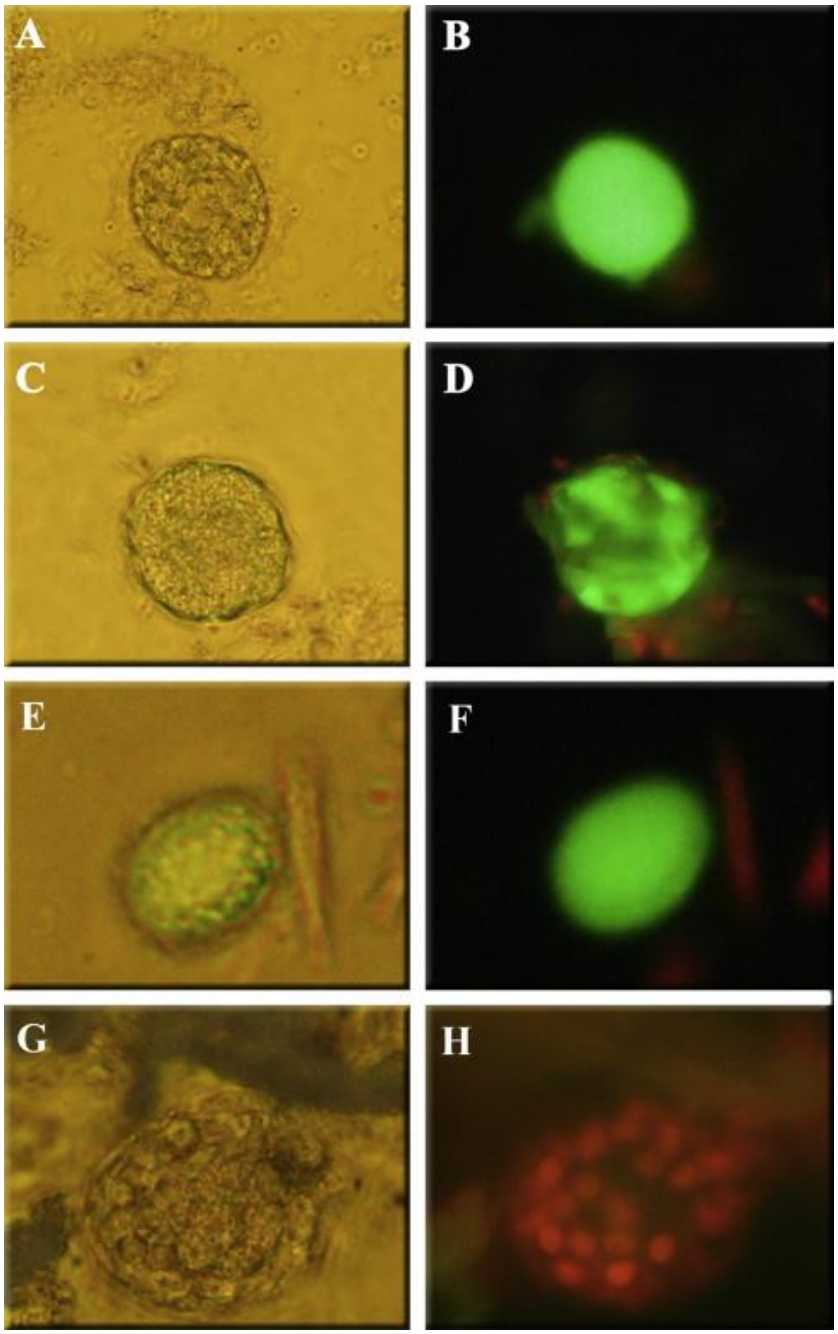


Fig. 7. Viability assessment of caprine preantral follicles using fluorescent probes. An isolated preantral follicle from fresh control (A and B), cultured for 14 d in medium containing follicle-stimulating hormone plus ascorbic acid (50 µg/mL) (C and D) or follicle-stimulating hormone alone (E and F), classified as viable since cells were labeled by calcein-AM (green fluorescence). Another follicle cultured in ascorbic acid (50 µg/mL) alone (G and H) was considered nonviable, as cells were marked with ethidium homodimer-1 (red fluorescence).

4. Discussion

This study provides the first demonstration of the positive influence of the combination of ascorbic acid and FSH on the survival and growth of caprine preantral follicles after long-term in vitro culture. The process of folliculogenesis in vivo in ruminants has long

been compared to that of rodents, and it can last about 4 to 6 months. Thus, it is very important to develop a long-term in vitro culture system that allows follicles to grow to more advanced stages, with the added benefit of providing a better understanding of ovarian physiology. In fact, few studies have demonstrated that long-term in vitro culture of preantral follicles enclosed in ovarian tissue promotes follicular activation in sheep [4] and humans [5] and [6]. To our knowledge, this is the first report that demonstrates the maintenance of caprine preantral follicle survival using histological and ultrastructural analysis, even after long-term culture (14 d) in domestic animals. Other studies related antrum formation in goats [28], cattle [29], and mice [30], and in the production of embryos [31] from in vitro culture of preantral follicles. It is important to mention that these studies used the large, advanced stage of secondary preantral follicles.

After 14 d of culture, addition of 50 µg/mL of ascorbic acid alone or in combination with FSH promoted an increase in the percentage of normal preantral follicles, compared with the culture in MEM. Ultrastructural analysis was performed in fresh control as well as in follicles cultured for 14 d in 50 µg/mL of ascorbic acid with or without FSH. After TEM, it was observed that only in fresh tissue or in culture with 50 µg/mL of ascorbic acid and FSH were important structures such as mitochondria, endoplasmic reticulum, and granulosa cells, as well as basal and nuclear membranes, preserved. The features observed in follicles were similar to those reported previously in the caprine species [21] and [32]. In addition to ultrastructural analysis, preantral follicles were further analyzed using a viability assay. The fluorescent probes (calcein-AM and ethidium homodimer-1) have been used successfully to assess the viability of bovine early-stage follicles [33]. In the present study, the results of this assay were similar to those observed using ultrastructural analysis, suggesting that TEM is a reliable method for viability assessment of preantral follicles. At physiological concentrations, ascorbic acid is an antioxidant that protects cells against the damage caused by reactive oxygen species (ROS) [7]. Among the actions of ascorbic acid are the prevention of DNA mutations induced by oxidation [9] and [10] and protection against lipidic peroxidation [11] and [12]. Similar to our results, Thomas et al. [14] verified the maintenance of follicular integrity and survival after in vitro culture of isolated bovine preantral follicles for 12 d in medium containing 50 µg/mL of ascorbic acid. In addition, Murray et al. [16] observed that ascorbic acid at this concentration promoted an increase in basement membrane remodeling and granulosa cell survival in isolated mouse preantral follicles cultured in vitro for 6 d.

Follicle-stimulating hormone alone maintained the percentage of normal follicles throughout the culture: from d 1 to d 7, and then out to d 14. Our histological results using light microscopy confirm earlier studies in which FSH maintained preantral follicle viability

after short- [21] or long-term culture [29], [34] and [35]. In addition, FSH positively regulates the absorption of ascorbic acid from granulosa cells [36], suggesting a possible beneficial effect of the interaction between these 2 substances. After 14 d of culture, the use of 100 μg/mL of ascorbic acid and FSH reduced the percentage of normal preantral follicles relative to the combination of 50 μg/mL of ascorbic acid and FSH. In fact, it is known that some antioxidant substances can act as pro-oxidants when used in high concentrations [37], suggesting that ascorbic acid at 100 μg/mL could be toxic to caprine preantral follicles cultured in vitro. Moreover, there are reports that high concentrations of ascorbic acid can inhibit important physiological processes in the ovary, resulting in follicular degeneration [16]. Furthermore, high concentrations of ascorbic acid can promote oxidative damage to cellular DNA, especially in the presence of Cu2+ and Fe2+ ions, which react with hydrogen peroxide and lead to the formation of the highly reactive hydroxyl radical that causes damage [15].

In this study, after 7 d of culture, FSH, ascorbic acid (50 μg/mL), and ascorbic acid at the concentrations of 50 and 100 μg/mL combined with FSH increased the percentage of follicular activation when compared to fresh control. In addition, over a culture period of 1 to 14 d, the use of FSH alone promoted the highest percentage of growing follicles. Ascorbic acid accumulates in the granulosa cells, inner theca, luteal cells, and oocytes [14]. Besides acting as an excellent antioxidant, it also positively influences the process of steroidogenesis [16] and stimulates follicular development [14]. Although some studies have suggested that follicular activation is an FSH-independent process [38] and [39], gonadotropins can promote early follicular development in vitro in ovine [40] and caprine cells [21]. Moreover, it has been demonstrated that FSH regulates the expression of several growth factors, such as kit ligand, growth and differentiation factor-9, and bone morphogenetic protein-15, which have important roles in the activation of primordial follicles and further follicular growth [41] and [42].

Compared with fresh control and MEM, 50 μg/mL ascorbic acid combined with FSH increases oocyte and follicular diameter after 7 d of culture. After 14 d, addition of 50 μg/mL of ascorbic acid increases only oocyte diameter. During follicular growth, ovulation and corpus luteum formation, the basement membrane and extracellular matrix undergo constant remodeling and therefore need a large amount of collagen. In this way, ascorbic acid acts intensively in follicular growth through its action in the biosynthesis of collagen, which influences the process of cellular membrane remodeling [15]. Regarding the role of FSH in growth, some studies report that FSH receptors are expressed in the granulosa cells from the primary stages onward [17] and [18]. Although FSH receptors are not present in primordial follicles, FSH seems to play an indirect role in early follicular development through the release

of paracrine factors produced by the larger follicles or by ovarian stroma cells [20]. In addition, FSH regulates the connection between the oocyte and the granulosa cell, facilitating the exchange of important substances for follicular growth and development [43].

It can be concluded that 50 μg/mL of ascorbic acid combined with FSH plays an important role in early folliculogenesis in goats, maintaining follicular survival and promoting the activation of primordial follicles and growth after long-term culture. The culture system described in our work may be very useful to evaluate the role of other substances (hormones and growth factors) on the in vitro development of early follicles.

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