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Ascorbic acid improves the survival and *in vitro* growth of isolated caprine preantral follicles

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Abstract

The present study aims to investigate the influence of two concentrations of ascorbic acid on the survival, growth, antral formation and mRNA expression of the matrix metalloproteinases-9 (MMP-9) and their tissue inhibitor-2 (TIMP-2) on caprine preantral follicles during long-term *in vitro* culture. Isolated preantral follicles were individually cultured without or with ascorbic acid at 50 µg/ml (AA50) or 100 µg/ml (AA100) during 18 days. The parameters evaluated were follicular viability, growth, antrum formation and extruded oocytes. The genes MMP-9 and TIMP-2 were quantified by real-time polymerase chain reaction (qPCR) after 18 days of culture in the control medium (MEM^+) or ascorbic acid (50 or 100 µg/ml) and in fresh control (non cultured). At the end of culture, AA50 significantly increased the percentage of viable follicles compared with other treatments. Moreover, mean daily increase in follicular diameter (µm/day) was significantly higher in the presence of both concentrations of ascorbic acid than in MEM^+ alone. Higher rates of antral formation and lower percentages of extruded oocytes were observed in medium containing AA50 compared with control medium. Real Time RT-PCR assays showed that AA50 increases MMP-9 expression significantly compared with fresh control and MEM^+ alone. In conclusion, ascorbic acid at 50 µg/ml was very important for the maintenance of caprine preantral follicle viability and development after *in vitro* culture and influences *in vitro* the enzymes involved with basement membrane remodeling.

Keywords: caprine, culture, MMP-9, preantral follicles, TIMP-2.

Introduction

The knowledge of the factors controlling the initiation and further development of preantral follicles is extremely limited, particularly in monovular species such as large domestic ruminants and humans in whom it takes several months for follicles to progress from initiation to the antral stage (Gougeon, 1996). Over recent years, one of the technologies that have attracted special attention of the researchers in the reproduction field is the *in vitro* culture of ovarian preantral follicles, which can act as a source of fertilizable oocytes for further *in vitro* embryo production (Gupta *et al*., 2007). In this way, several culture systems of large secondary follicles have been developed in different species, resulting in the formation of the antral cavity (ovine: Cecconi *et al*., 1999; bovine: Gutierrez *et al*., 2000; caprine: Huamin and Yong, 2000), oocyte maturation (ovine: Tamilmani *et al*., 2005) and, more recently, embryo production (bubaline: Gupta *et al*., 2008). Additionally, *in vitro* maturation (IVM) and subsequent *in vitro* fertilization (IVF) of oocytes from mouse preantral follicles have resulted in the birth of live offspring (O'Brien *et al*., 2003).

The maintenance of oocyte-granulosa cells tridimensional structure throughout the culture is essential for normal follicular differentiation as well as for the production of an oocyte competent to undergo fertilization and embryogenesis (Eppig, 2001). Consequently, *in vitro* culture systems of preantral follicles should maintain viability and promote growth during long periods of culture. Therefore, several substances have been added to the culture medium with the aim of improving these systems (Eppig *et al*., 2000; Gutierrez *et al*., 2000; Mao *et al*., 2004). Among them, ascorbic acid is a dietary requirement vitamin for primates and a few other mammals (Thomas *et al*., 2001).

Ascorbic acid is an antioxidant widely distributed in animal tissues and is found in the pituitary, adrenal and gonads (Luck *et al*., 1995). In the mammalian ovary, this substance is found in granulosa cells, inner theca, as well as in the oocyte (Thomas *et al*., 2001). This antioxidant is produced by hepatic synthesis in species such as rodents and domestic animals; however, it must be available in the diet of primates and guinea pigs. The vitamin is then transported from blood into the peripheral tissues and accumulated via membrane transporters against a large concentration gradient, most notably in the adrenal glands and the gonads, which contain high concentrations of ascorbic acid (Zreik *et al*., 1999).

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In animal reproduction, ascorbic acid has been implicated in the biosynthesis of collagen, thus being important during follicular growth, ovulation and corpus luteum formation. Additionally, it acts in the prevention and reduction of biomolecular oxidation (Sebrell and Harris, 1967). Moreover, the presence of high concentrations of ascorbic acid in endocrine tissues is thought to be important for the production of steroid hormones (Tsuji *et al*., 1989) and apoptosis inhibition in bovine granulosa cells (Tilly and Tilly, 1995) and mouse cumulus-oocyte-complex (COC; Eppig *et al*., 2000, Murray *et al*., 2001). Furthermore, Tatemoto *et al.* (2001) verified that addition of ascorbic acid to the maturation medium protected swine oocytes from oxidative stress and increased the formation of pronucleus, further cleavage and blastocyst development. In this way, the antioxidant property of ascorbic acid in the culture medium can reduce the damage of reactive oxygen species (ROS; forming the ascorbate as a stable free radical; Buettner, 1993), and metal-oxygen complexes to DNA, proteins, carbohydrates, lipids and cell membranes (Sies *et al.*, 1992).

Adequate turnover of components of the extracellular matrix of the follicle is essential for normal follicle growth, follicle repair after ovulation (Himeno *et al*., 1984) and subsequent development of the corpus luteum (Luck and Zhao, 1993). The matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of metalloproteinases (TIMPS), are members of an enzyme family associated with the turnover of collagen on the basement membrane during follicle growth. A serum-free culture system for bovine preantral follicles was used to show that MMP and TIMP activities are secreted *in vitro*, and that MMP-9 and TIMP-2 are markers of follicle health (McCaffery *et al*., 2000). Ascorbic acid may act to regulate these factors (Murray *et al*., 2001; Thomas *et al*., 2001). However, there are no reports on the possible role of ascorbic acid addition on the caprine preantral follicle survival and development *in vitro*.

The present study aims to investigate the influence of two concentrations of ascorbic acid on the survival, growth and antrum formation of caprine preantral follicles during long-term *in vitro* culture. Moreover, the effect of this antioxidant on the mRNA expression of the MMP- 9 and TIMP- 2 was analyzed.

Materials and Methods

Chemicals

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

Source of ovaries

Ovaries (n = 100) from 50 adult (1 - 3 years old), mixed-breed goats were collected at a local slaughterhouse. The surrounding fat tissue and ligaments were removed and the ovaries were washed in 70% alcohol followed by two washes in Minimum Essential Medium (MEM). The ovaries were placed into tubes containing 15 ml of MEM plus HEPES (MEM HEPES), supplemented with 100 μg/ml penicillin and 100 μg/ml streptomycin and then transported to the laboratory in MEM at 4°C (Chaves *et al*., 2008) within 1 h.

Isolation and selection of caprine preantral follicles

In the laboratory, the surrounding fat tissue and ligaments were removed from the ovaries. Ovarian cortical slices (1 mm thick) were cut from the ovarian surface using a surgical blade under sterile conditions. Then the ovarian cortex tissues were placed and washed in fragmentation medium which consisted of MEM HEPES. Preantral follicles of approximately ≥150 μm in diameter were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and manually dissected from the strips of ovarian cortex using 26 G needles and then transferred to the culture medium. Follicles with a visible central oocyte, surrounded by two or more granulosa cell layers, an intact basement membrane and no antral cavity were selected for culture.

Caprine preantral follicle culture

After selection, follicles were individually cultured in 25 μl drops of culture medium under mineral oil in Petri dishes (60 x 15 mm, Corning, USA). Basic culture medium consisted of α-MEM (pH 7.2 - 7.4) supplemented with 1.25 mg/ml bovine serum albumin (BSA), ITS (insulin 10 μg/ml, transferrin 5.5 μg/ml and selenium 5 ng/ml), 2 mM glutamine and 2 mM hypoxantine (control: MEM^{+}). Follicles were randomly distributed in each of the following treatments: control (MEM^{+}) without or with ascorbic acid at 50 μg/ml (AA50) or 100 μg/ml (AA100). Incubation was carried out at 39°C and 5% CO_2 in air during 18 days. Fresh media were prepared before use and incubated for 1 h prior to use. Every other day, 15 μl of the culture media were replenished by fresh medium. The culture was replicated four times, and at least 32 follicles were used per treatment.

Morphological evaluation of follicle development

After culture, follicles were classified according to their morphology. A follicle was considered normal when presented a centrally located spherical and homogeneous oocyte, surrounded by two or three compact layers of granulosa cells, and with no apparent damage to the basement membrane in the beginning of culture. Those follicles that showed morphological signs of degeneration, such as darkness of oocytes and surrounding cumulus cells or those with misshapen oocytes, were considered degenerated. Follicular diameter was measured only in healthy

follicles every 6 days with the aid of an ocular micrometer inserted into a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan; 100X magnification). The oocyte diameter was measured at the end of the culture period. Both diameters, from the basement membrane at right angles (90°) to each other in the largest cross-section of each growing oocyte and follicle were measured and averaged. Two diameters were recorded for each and the average of these two values was reported as follicle and oocyte diameters, respectively. Antral cavity formation was defined as a visible translucent cavity within the granulosa cell layers and extrusion of oocytes from the follicles was also recorded. Regarding the follicular growth, the mean increase in follicular diameter was calculated as follows: diameter of viable follicles at day 18 minus diameter of viable follicles at day 0, divided by total number of viable follicles at day 18.

Recovery rate and in vitro maturation of caprine oocytes from in vitro cultured follicles

At day 18 of culture, all healthy follicles were carefully and mechanically opened with 26 G needles under 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 recovery rate was calculated by dividing the number of oocytes ≥110 µm by the number of viable follicles at day 18 of culture x 100. The selected COCs were washed three times in maturation medium composed of TCM199 supplemented with 10% fetal calf serum, 100 µg/ml of Luteinizing Hormone (LH), 5 µg/ml of rFSH, 10 ng/ml epidermal growth factor and 1 µg/ml 17β-estradiol. After washing, the oocytes were transferred to 50 µl drops of maturation medium, under mineral oil and then incubated for 26 h at 39ºC with 5% CO_2 in air. At the end of the maturation period, oocytes were assessed for nuclear maturation following Hoechst staining.

Viability assessment of follicles cultured in vitro

For a better evaluation of follicular integrity, after 18 days of culture, live/dead fluorescent staining was performed on isolated goat preantral follicles (n = 10 for each treatment) in 100 µl droplets of MEM mounted in glass slides with 4 µm calcein-AM and 2 µm ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany), followed by an incubation at 37°C for 15 min and finally the follicles were examined using a DMLB fluorescence microscope (Leica, Germany). The emitted fluorescent signals of calcein-AM and ethidium homodimer were collected at 488 and 568 nm, respectively. While the first probe detected intracellular esterase activity of viable cells, the later labeled nucleic acids of non-viable cells with plasma membrane disruption. 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).

MMP-9 and TIMP-2 mRNA expression

For RNA isolation, 3 pools of 10 follicles from non cultured (fresh control), MEM^+ alone, AA50 and AA100) were collected. Total RNA was isolated with TRIzol Plus Purification kit (Invitrogen, São Paulo, Brazil).

The RNA preparations were submitted to a DNase I treated with an RNeasy Micro kit (Invitrogen). Complementary DNA (cDNA) was synthesized from the RNA (0.15 µg from each sample) using Superscript™ II Rnase H-Reverse Transcriptase (Invitrogen Life Technologies).

The PCR reaction was carried out in a final volume of 20 µl containing: 1 µl of each cDNA, 1x Power SYBR® Green PCR Master Mix (10 µl), 7.4 µl of ultra-pure water and 0.4 µm of both sense and antisense primer. Gene-specific primers for amplification of different transcripts were shown in Table 1. Samples were first denaturated at 95°C for 10 min, then submitted to 40 PCR cycles (15 sec 95°C, 1 min 60°C, 1 min 72°C), followed by a final extension of 10 min at 72°C. The specificity for each primer set was tested with a melt curve, carried out between 60 and 95°C for all genes. All amplifications were carried out in Bio-Rad iQ5. The delta-delta-CT method was used to transform CT values into normalized relative expression levels.

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

Target gene	Primer sequence (5´→ 3´)	Sense	Position	GenBank accession nº
GAPDH	TGTTTGTGATGGGCGTGAACCA	S	287-309	GI: 27525390
	ATGGCGTGGACAGTGGTCATAA	As	440-462	
β-Actin	ACCACTGGCATTGTCATGGACTCT	S	187-211	GI: 28628620
	TCCTTGATGTCACGGACGATTTCC	As	386-410	
MMP-9	TTTCCTCCTGGCTCAGGCATTCA	S	21-44	GI: 206573538
	GTTTCCGAAGTAGGTCGGGATCACA	As	119-144	
TIMP-2	AGAAGAAGAGCCTGAACCACAGGT	S	419-443	GI: 261244947
	TGATGTTCTTCTCCGTGACCCAGT	As	550-574	

s, sense; as, antisense.

Statistical analyses

The percentage of follicular survival, antrum formation, extrusion rate and maturation after *in vitro* culture were compared by chi-square test (StatView for Windows). Follicular diameter and development after culture were compared by ANOVA and Kruskal-Wallis test. For Real Time RT-PCR treatment and control samples were randomly assigned in blocks and the relative expression values ($2^{-\Delta\Delta Ct}$) were subjected to Shapiro-Wilk normality test using the UNIVARIATE procedure of SAS 9.0 software package. The relative expression was logarithmically transformed ($\log_{10}(X + 1)$) for normal distribution adjustment. Log-transformed relative expression was evaluated by analysis of variance using the ANOVA procedure and statistical significance of the differences between the control and treatments was assessed with the T test ($P < 0.05$) of SAS 9.0.

Results

Effect of ascorbic acid on follicle survival

Preantral follicles selected for culture had a centrally located oocyte and normal granulosa cells, which were enclosed by an intact basement membrane (Fig. 1A). As shown in Fig. 2, comparisons performed among the treatments in the same culture period demonstrated that there were no significant differences up to 6 days of culture regarding the percentage of normal follicles. However, after 12 and 18 days, AA50 significantly increased the percentage of normal follicles compared with other treatments ($P < 0.05$). At the end of culture, medium without ascorbic acid had the lowest percentage of normal follicles ($P < 0.05$). As the culture period progressed, there was a significant reduction in normal follicles in all treatments ($P < 0.05$).

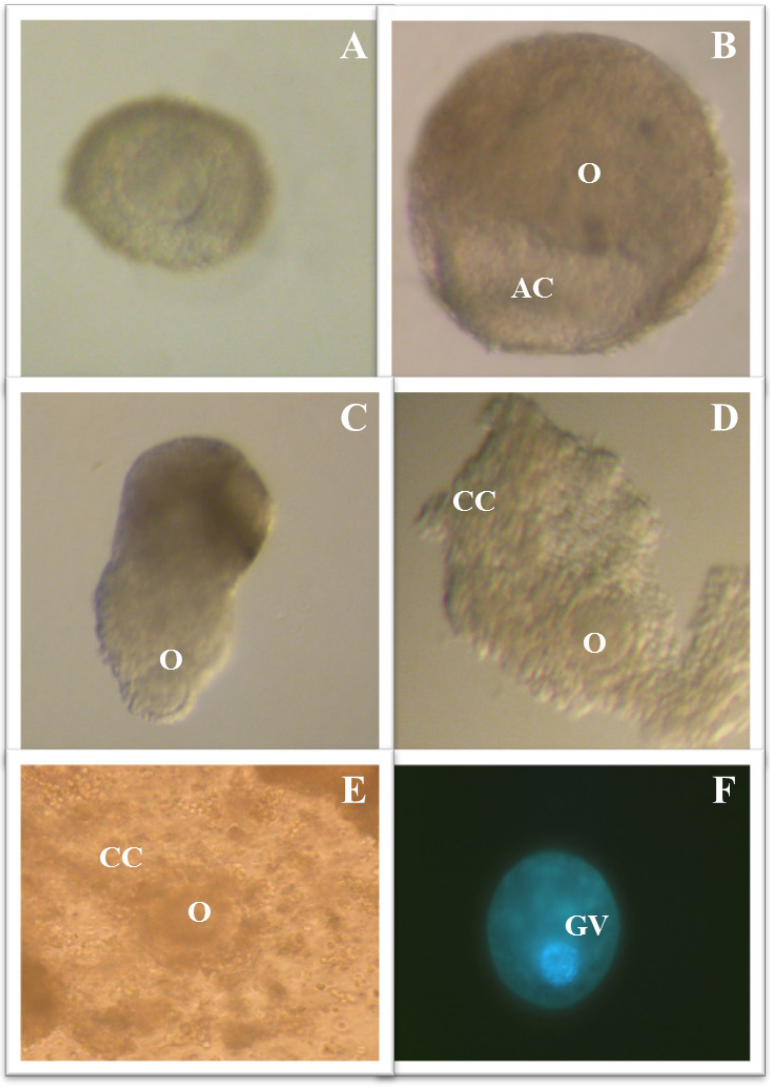

Figure 1. (A) Preantral follicles before culture (Day 0); (B) Preantral follicles after 2 days of culture in medium containing 50 µg/ml of ascorbic acid; (C) Extrusion of oocyte from the follicle after culture with control medium; (D) Cumulus-oocyte complex recovered after 18 days of culture with 50 µg/ml of ascorbic acid; (E) Expanded cumulus cells visualized in some COC; (F) Intact germinal vesicle. GC: Granulosa cells, TC: theca cells, O: oocyte, AC: antral cavity, GV: germinal, vesicle; CC: cumulus cells.

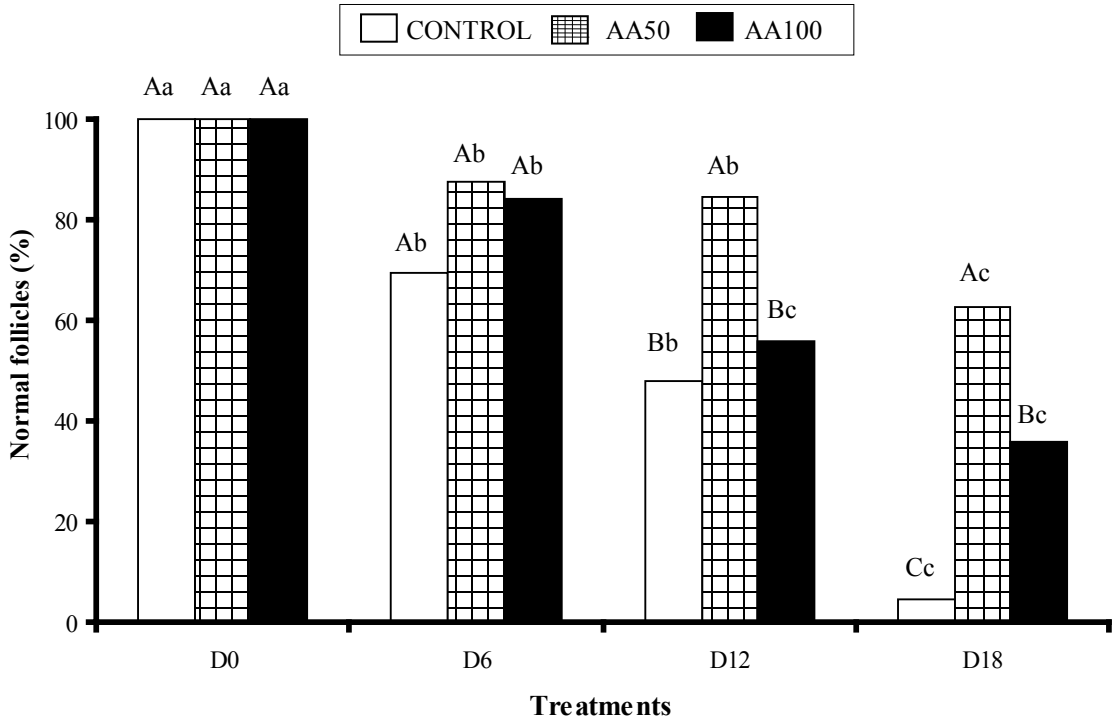

Figure 2. Percentage of morphologically normal preantral follicles cultured for 18 days in control medium (MEM^+) or ascorbic acid (50 or 100 µg/ml). ^{A,B}Different letters denote significant differences among treatments in the same period ($P < 0.05$). ^{a,b}Different letters denote significant differences among culture periods within the same treatment ($P < 0.05$).

Effects of ascorbic acid on follicular growth and antral cavity formation

Mean initial diameter of follicles before culture (Day 0) was 168.5 ± 39.2, 184.6. ± 39.2 and 175.1 ± 33.8 for MEM^+, AA50 and AA100, respectively, and there was no significant difference among them. As shown in Fig. 3, after 6 days of culture, AA50 significantly increased follicular diameter, compared with the other treatments ($P < 0.05$). At day 12, both concentrations of ascorbic acid significantly increased follicular diameter when compared with control medium ($P < 0.05$). However, after 18 days of culture, there was no significant difference among all treatments regarding follicular growth.

Additionally, preantral follicles cultured in control medium (MEM^+) only showed a significant increase in follicular diameter in the beginning of the culture, i.e., from day 0 to 6 ($P < 0.05$). However, both concentrations of ascorbic acid promoted a progressive and significant increase in diameter of the follicles from day 0 to 6 and from day 6 to 12 of culture ($P < 0.05$). After 18 days, the final diameters observed were 517.9 ± 181.2 and 619.9 ± 246.5 µm for follicles cultured in AA50 and AA100, respectively. Moreover, mean daily (µm/day) increase in follicular diameter, after 18 days of *in vitro* culture was significantly higher in the presence of ascorbic acid (AA50: 18.1 ± 510.6 µm/day and AA 100: 20.9 ± 10.4 µm/day; $P < 0.05$) than in MEM^+ (11.4 ± 9.5 µm).

The first antral follicles were observed as early as day 2 of culture in follicles cultured with 50 µg/ml of ascorbic acid (Fig. 1B). The percentage of antral cavity formation after culture of preantral follicles in two concentrations of ascorbic acid is shown (Fig. 4). It is important to note that at day 6, follicles from all treatments (including control medium) reached the antral stage. After 12 and 18 days, the percentage of antrum development was similar between both ascorbic acid concentrations; however, AA50 increased antral cavity formation compared with control medium (MEM^+; 68.7 x 29.0% for AA50 and MEM^+, respectively; $P < 0.05$).

Figure 5 shows the percentage of extruded oocytes from the follicles, which was first observed after 6 days of culture in all treatments ($P < 0.05$). This extrusion (Fig. 1C) was characterized by rupture of the basement membrane with further compromising of follicular integrity and survival. After 12 days, addition of ascorbic acid at 50 µg/ml to the culture medium significantly reduced the percentage of extruded oocytes, compared with control medium and AA100 ($P < 0.05$). In addition, at day 18, both concentrations of ascorbic acid had similar percentage of extruded oocytes.

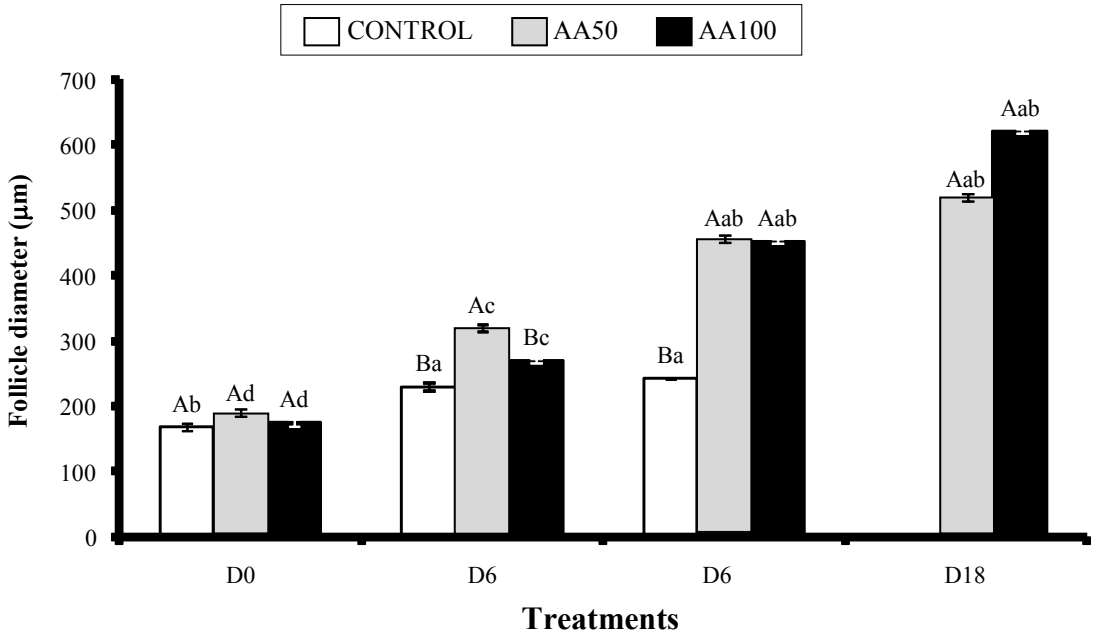

Figure 3. Diameter of follicles cultured for up to 18 days in control medium (MEM$^+$) or ascorbic acid (50 or 100 µg/ml). Results are presented as mean ± SEM. ^{A,B}Different letters denote significant differences among treatments in the same period ($P < 0.05$). ^{a,b}Different letters denote significant differences among culture periods within the same treatment ($P < 0.05$).

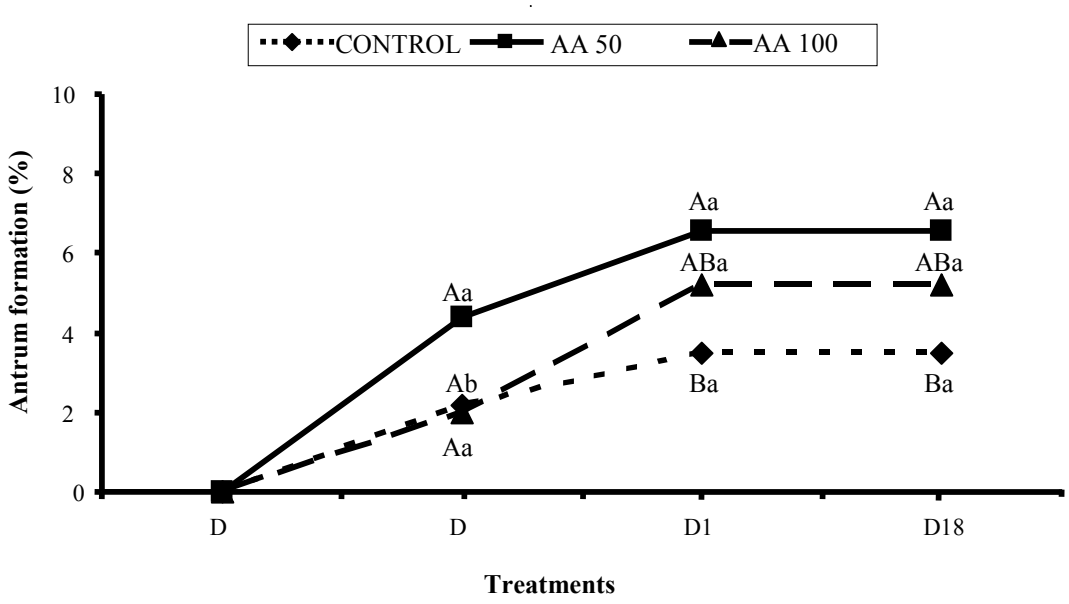

Figure 4. Percentage of antral cavity formation of preantral follicles cultured for 18 days in control medium (MEM$^+$) or ascorbic acid (50 or 100 µg/ml). ^{A,B}Different letters denote significant differences among treatments in the same period ($P < 0.05$). ^{a,b}Different letters denote significant differences among culture periods within the same treatment ($P < 0.05$).

Viability assessment of follicles cultured with ascorbic acid

A fluorescence cell viability assay based on labelling of live and dead cells by calcein-AM and ethidium homodimer-1, respectively, was employed to confirm follicle integrity after *in vitro* culture for 18 days. Only caprine preantral follicles cultured with both concentrations of ascorbic acid remained viable as assessed by calcein-AM/ethidium homodimer assays (Fig. 6).

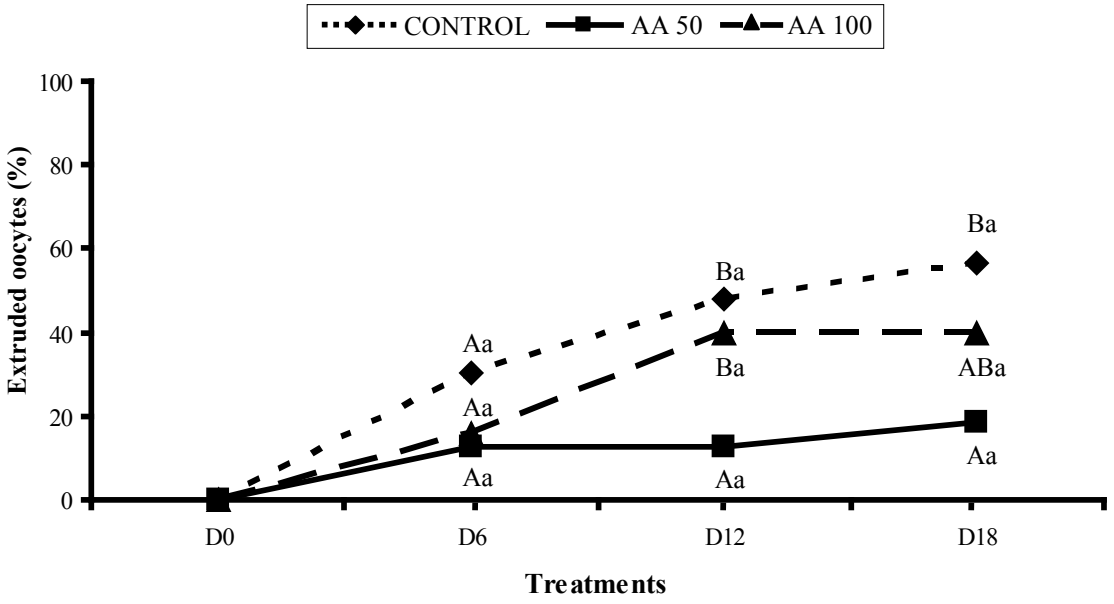

Figure 5. Percentage of extruded oocytes after 18 days of culture in control medium (MEM^{+}) or ascorbic acid (50 or 100 µg/ml). ^{A,B}Different letters denote significant differences among treatments in the same period ($P < 0.05$). ^{a,b}Different letters denote significant differences among culture periods within the same treatment ($P < 0.05$).

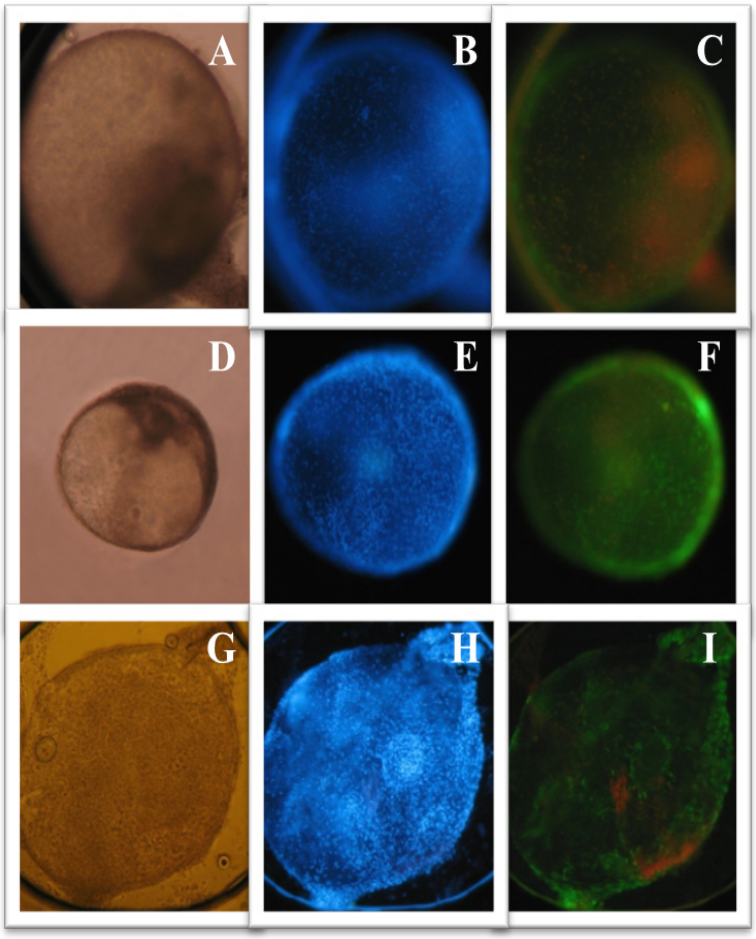

Figure 6. Fluorescence cell viability assay after 18 days of in vitro culture in control medium (A, B and C), ascorbic acid 50 µg/ml (D, E and F) and ascorbic acid 100 µg/ml (G, H and I). White field (A, D and G); Follicle labeled with HOESCHT (B, E and H); Follicle labeled with calcein (C, F and I).

Recovery rate of fully grown oocytes after in vitro culture

In regard to the percentage of oocytes destined to IVM, at the end of the culture period (after 18 days), 0, 18.7 and 28.0% of the oocytes (≥110 μm) were obtained from follicles cultured with MEM^+, AA50 and AA100, respectively. These oocytes had homogeneous cytoplasm and were surrounded by at least one compact layer of cumulus cells, characterizing the COC, as shown in Fig. 1D. At the end of the maturation period, expanded cumulus cells were visualized in two COCs (Fig. 1E). With respect to the chromatin configuration, all fully grown oocytes from follicles cultured with both ascorbic acid concentrations showed an intact germinal vesicle (Fig. 1F).

Effect of ascorbic acid on the MMP-9 and TIMP-2 mRNA expression

The results of qPCR analysis of the MMP-9 and TIMP-2 representative genes are shown (Fig. 7). A tendency for increase of expression of these genes with the *in vitro* culture could be observed. The expression of mRNA for MMP-9 was not detectable in the fresh control. AA50 and AA100 increased mRNA expression for MMP-9 when compared with fresh control and MEM^+ group ($P < 0.05$). In addition, mRNA expression for TIMP-2 gene was greater in AA100 than in MEM^+ ($P < 0.05$). However, there was no significant difference among AA50 and all treatments and between AA100 and fresh control.

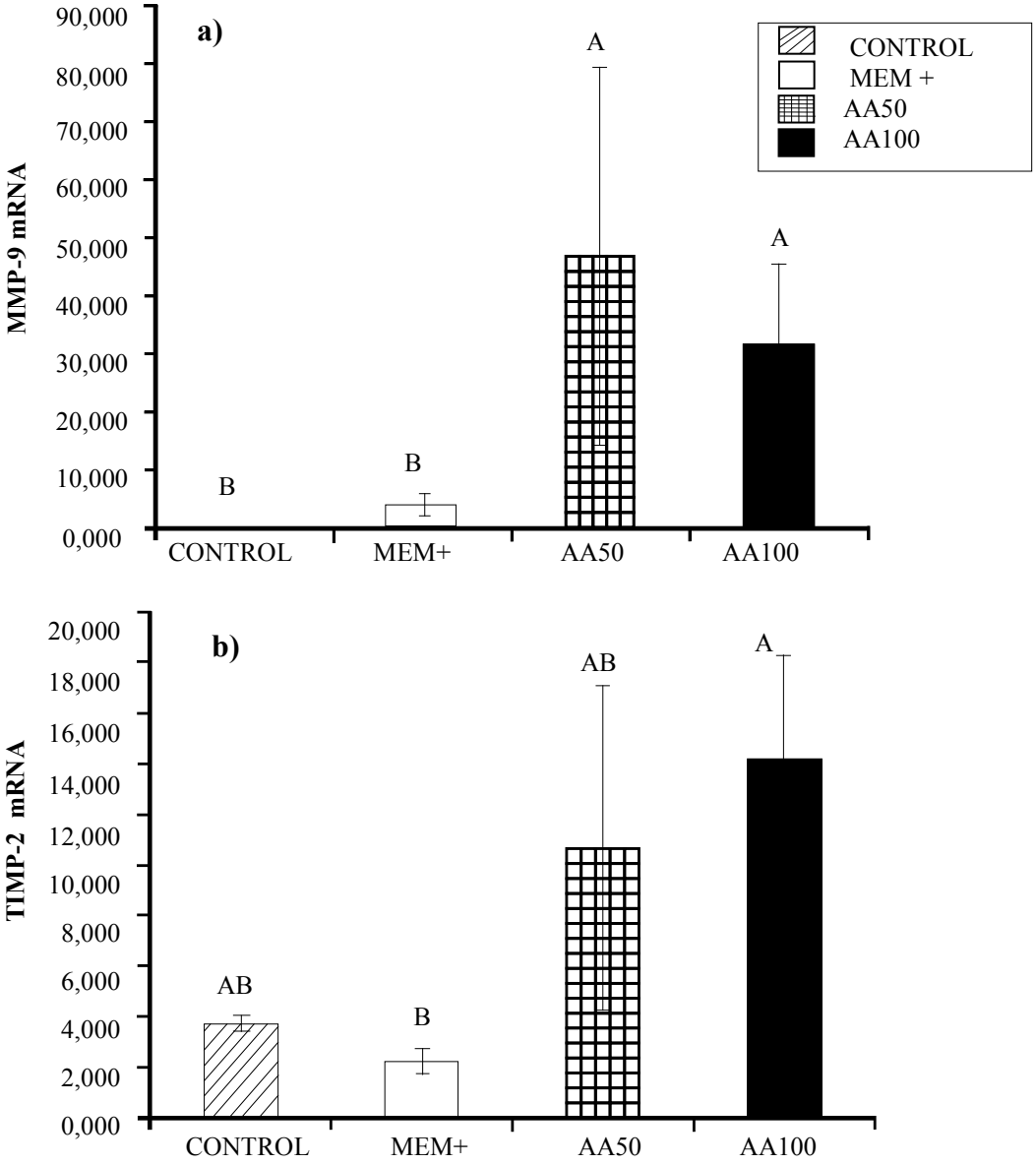

Figure 7. Expression of mRNA of (a) MMP-9 and (b) TIMP-2 after 18 days of culture in the control medium (MEM^+) or ascorbic acid (50 or 100 μg/ml) and in fresh control (non cultured). Results are presented as mean ± SEM. $^{A, B}$Different superscripts denote statistically different values ($P < 0.05$).

Discussion

The present study demonstrated the importance of ascorbic acid on the viability, growth, antrum formation and regulation of the enzymes MMP-9 and TIMP-2 involved in remodeling of the extracellular matrix of caprine preantral follicles after long-term culture *in vitro*. The concentrations of ascorbic acid used in this experiment (50 and 100 µg/ml) were based on previous studies performed with other species (bovine: Thomas *et al*., 2001; murine: Rose *et al*., 1999; swine: Mao *et al*., 2004).

In this study, after 18 days of culture, treatment with AA50 showed higher rates of follicular survival than control and AA100. Nevertheless, in the same period, only a small percentage of viable follicles (4.3%) were observed in control medium (MEM^+), thus confirming the importance of adding ascorbic acid to the culture medium. Previous studies demonstrated that ascorbic acid is predominantly a soluble antioxidant in the ovary, which significantly decreases preantral (mouse: Murray *et al*., 2001) and antral (rats: Tilly and Tilly, 1995) follicle apoptosis up to 6 days of culture. Moreover, addition of ascorbic acid to the medium promoted the maintenance of secondary follicles in mouse and cows (Rose *et al*., 1999; Thomas *et al*., 2001). Other antiapoptotic effects of ascorbic acid were verified in the culture of rat luteinic cells (Kolodecik *et al*., 1998) and co-culture of oocytes with granulosa cells (mouse; Eppig *et al*., 2000). Generally, antioxidants protect plasmatic membranes by removing ROS, thus reducing their damage (Alvarez and Moraes, 2006). Additionally, the progression of the culture may cause the production of toxic metabolites and ROS, such as H_2O_2 and O_2^-, which may cause oxidation of key molecules that normally release proteases, lipases, and nucleases from mitochondria (Fiers *et al*., 1999), which probably lead to follicular degeneration. In the present study, the use of ascorbic acid in the medium may have reduced ROS production.

Additionally, at day 18, both concentrations of ascorbic acid have similar low percentages of extruded oocytes, characterized by rupture of the basement membrane with further loss of follicular integrity. In contrast, medium without ascorbic acid showed a higher percentage of extrusion of the oocytes from the follicles. Similar to our results, Thomas *et al*. (2001) demonstrated that ascorbic acid maintains the integrity of bovine follicles cultured in medium without fetal serum, and that addition of this vitamin reduces cell death, contributing to extracellular matrix (ECM) turnover. In a mouse model, Murray *et al*. (2001) verified that ascorbic acid maintains the percentage of follicles with intact basement membrane. This fact can be due to the role of ascorbic acid in the production of collagen type IV, which is important to conserve follicular stability, in addition to prevent the lack of basement membrane integrity (Luck *et al*., 1995). Thus, in our study, the integrity of caprine follicles observed with addition of ascorbic acid to the medium may be due to its possible influence on the maintenance of gap junctions (Amsterdam and Rotmensch, 1987). The gap junctions are channels between the oocyte and granulosa cells and cumulus enabling nutritional support, transmission of electrical signals and transport of messenger molecules (Kalvelyte *et al*., 2003). Their maintenance is essential for obtaining good follicle culture *in vitro* results.

To date, little is known about the effect of ascorbic acid on preantral follicle growth. This growth is followed by a constant turnover of the basement membrane, and the layers of granulosa and theca cells which surround the oocyte (Gutierrez *et al*., 2000). In the current study, both concentrations of ascorbic acid positively influenced the increase of follicular diameter, compared with the control group. Conversely, other authors did not observe any influence of this substance on follicular diameter (Murray *et al*., 2001; Thomas *et al*., 2001). These contradictory results may be due to the differences in the methodologies and species studied. The fact that ascorbic acid can act as a co-factor in collagen synthesis and peptidic amidation, facilitating follicular development (Christiane *et al*., 1988) by an adequate turnover of the basement membrane, allowing for follicular expansion, may explain the positive effects of this antioxidant on caprine preantral follicle growth observed in our study. As early as day 6, all treatments showed some follicles with antral cavity, although the highest rates of antrum formation were observed in treatments containing ascorbic acid, especially 50 µg/ml. This may be due to the beneficial effects of ascorbic acid on the preservation of basement membrane integrity (low rates of extruded oocytes), follicular viability and growth.

This is the first study about the influence of ascorbic acid on the mRNA expression of MMP-9 and TIMP-2 on isolated caprine preantral follicles. The present study showed that treatment with AA50 increases the expression of MMP-9. This was the same treatment in which a significantly lower percentage of extruded oocytes were observed. Moreover, both concentrations of this antioxidant influence TIMP-2 expression. MMPs are considered to be the most important enzymes for tissue degradation (Woessner, 1991). They are regulated by TIMPs and possess the ability to degrade basement membrane collagens and denatured collagen within the follicle. During follicular growth and expansion there is rapid production of the basal lamina that separates the thecal and granulosa compartments. This has been estimated as a 3×10^4 times increase in mice (Gosden *et al*., 1993). In the *in vitro* system described in the present study, caprine follicles cultured in MEM^+, AA50 and AA100 grew from approximately 170-230 µm 185-520 µm and 176-620 µm, respectively, resulting in a 1.4, 2.8 and 3.5 times increase in follicular surface area. Therefore, to

maintain expansion of this membrane during development, it could be assumed that the growing follicle would have a high requirement for ascorbic acid that influence the enzymes involved in remodelling basal lamina. Results from the present study are in agreement with other studies *in vitro* (Rose *et al*., 1999; Murray *et al*., 2001; Thomas *et al*., 2001), which have demonstrated promotion of follicle integrity and survival by increase in MMP and TIMP activity in the presence of ascorbic acid in cultured mouse and bovine follicles. Those authors emphasize the usefulness of MMP detection as a means of monitoring follicle health throughout culture.

Previous studies demonstrated that caprine oocytes smaller than 100 µm were not able to resume meiosis (Crozet *et al*., 2000). Based on that, in our study, at the end of culture only fully grown oocytes larger than 100 µm from viable follicles were used for IVM procedure. In this study, *in vitro* culture of caprine preantral follicles in the presence of ascorbic acid was better for oocyte growth since we achieved 0, 18.7 and 28.0% of fully grown oocytes in MEM^{+}, AA50 and AA100, respectively, after 18 days of culture. However, after IVM, all fully grown oocytes from follicles cultured with both ascorbic acid concentrations did not resume meiosis and showed an intact germinal vesicle. Few studies in domestic animals have achieved meiotically competent oocytes from the *in vitro* culture of preantral follicles (ovine: Tamilmani *et al.*, 2005; Arunakumari *et al*., 2007; swine: Wu *et al*., 2006; bubaline: Gupta *et al*., 2008). The absence of meiotic resumption obtained in our study was probably due to the lack of growth factors and/or hormones in the culture medium, which was insufficient to promote the *in vitro* development of caprine oocytes capable to resume meiosis.

In conclusion, ascorbic acid at 50 µg/ml was very important for the maintenance of caprine preantral follicle viability and development after *in vitro* culture. In addition, ascorbic acid increased the production of MMP-9 and TIMP-2 by the cultured follicles, which implicates the effect of this vitamin in regulation of MMP remodeling of the basement membrane. Addition of hormones and growth factors to the culture medium described in the present study may be important in the future for the production of potentially viable oocytes, which can be destined for maturation, fertilization and further *in vitro* embryo production.

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References

Alvarez CA, Moraes GV. 2006. Efeitos da selenometionina e vitamina C sobre o sêmen. *Saúde Biol*, 1:42-51.

Amsterdam A, Rotmensch S. 1987. Structure-function relationships during granulosa cell differentiation. *Endocr Rev*, 8:309-337.

Arunakumari G, Vagdevi R, Rao BS, Naik BR, Naidu KS, Suresh Kumar RV, Rao VH. 2007. Effect of hormones and growth factors on in vitro development of sheep preantral follicles. *Small Rumin Res,* 70:93-100.

Buettner GR. 1993**.** The pecking order of free radicals and antioxidants: lipid peroxidation, α-tocopherol, and ascorbate. *Arch Biochem Biophys*, 300:535-543.

Cecconi S, Barboni B, Coccia M, Mattioli M. 1999. In vitro development of sheep preantral follicles. *Biol Reprod*, 60:594-601.

Chaves RN, Martins FS, Saraiva MV, Celestino JJ, Lopes CA, Correia JC, Verde IB, Matos MH, Báo SN, Name KP, Campello CC, Silva JR, Figueiredo JR. 2008. Chilling ovarian fragments during transportation improves viability and growth of goat preantral follicles cultured in vitro. *Reprod Fertil Dev*, 20:640-647.

Christiane Y, Demoulin A, Gillain D, Leroy F, Lambotte R, Lapiere CM, Nusgens B, Foidart J-M. 1988. Laminin and type III procollagen peptide in human preovulatory follicular fluid. *Fertil Steril*, 50:48-51.

Crozet N, Dahirel M, Gall L. 2000. Meiotic competence of in vitro grown goat oocytes. *J Reprod Fertil*, 118:367-373.

Eppig JJ, Hosoe M, O'Brien MJ, Pendola FM, Requena A, Watanabe S. 2000. Conditions that affect acquisition of developmental competence by mouse oocytes in vitro: FSH, insulin, glucose and ascorbic acid. *Mol Cell Endocrinol*, 163:109-116.

Eppig JJ. 2001. Oocyte control of ovarian follicular development and function in mammals. *Reproduction*, 122:829-838.

Fiers W, Beyaert R, Declercq W, Vandenabeele P. 1999. More than one way to die: apoptosis, necrosis and reactive oxygen damage. *Oncogene*, 18:7719-7730.

Gosden RG, Boland NI, Spears N, Murray AA, Chapman M, Wade JC, Zhody NI, Brown N. 1993. The biology and technology of follicular oocyte development in vitro. *Reprod Med Rev,* 2:129-152.

Gougeon A. 1996. Regulation of ovarian follicular development in primates: facts and hypotheses. *Endocr Rev*, 17:121-155.

Gupta PSP, Rameshb HS, Nandi S, Ravindra JP. 2007. Recovery of large preantral follicles from buffalo ovary: effect of season and corpus luteum. *Anim Reprod Sci*, 101:145-152.

Gupta PSP, Ramesh HS, Manjunatha BM, Nandi S, Ravindra JP. 2008. Production of buffalo embryos using oocytes from *in vitro* grown preantral follicles. *Zygote*, 16:57-63.

Gutierrez CG, Ralph JH, Telfer EE, Wilmut I, Webb R. 2000. Growth and antrum formation of bovine preantral follicles in long term culture *in vitro*. *Biol*

Reprod, 62:1322-1328.

Himeno N, Kawamura N, Okamura H, Mori T, Fukumoto M, Midorikawa O. 1984. Collagen synthetic activity in rabbit ovary during ovulation and its blockage by indomethacin. *Nippon Sanka Fujinka Gakkai Zasshi*, 36:1930-1934.

Huamin Z, Yong Z. 2000. In vitro development of caprine ovarian preantral follicles. *Theriogenology*, 54:641-650.

Kalvelyte A, Imbrasaite A, Bukauskiene A, Verselis VK, Bukauskas FF. 2003. Connexins and apoptotic transformation. *Biochem Pharmacol*, 66:1661-1672.

Kolodecik TR, Aten RF, Behrman HR. 1998. Ascorbic acid-dependent cytoprotection of ovarian cells by leukocyte and nonleukocyte peroxidases. *Biochem Pharmacol*, 55:1497-1503.

Luck MR, Zhao Y. 1993. Identification and measurement of collagen in the bovine corpus luteum and its relationship with ascorbic acid and tissue development. *J Reprod Fertil,* 99:647-652.

Luck MR, Jeyaseelan I, Scholes RA. 1995. Ascorbic acid and fertility. *Biol Reprod*, 52:262-266.

Mao J, Smith MF, Rucker EB, Wu GM, McCauley TC, Cantley TC, Prather RS, Didion BA, Day BN. 2004. Effect of epidermal growth factor and insulin-like growth factor I on porcine preantral follicular growth, antrum formation, and stimulation of granulosal cell proliferation and suppression of apoptosis in vitro. *J Anim Sci*, 82:1967-1975.

McCaffery FH, Leask R, Riley SC, Telfer EE. 2000. Culture of bovine preantral follicles in a serum-free system: markers for assessment of growth and development. *Biol Reprod,* 63:267-273.

Murray A, Molinek MD, Baker SJ, Kojima FN, Smith MF, Hillier SG, Spears N. 2001. Role of ascorbic acid in promoting follicle integrity and survival in intact mouse ovarian follicles in vitro. *J Reprod Fertil*, 121:89-96.

O'Brien MJ, Pendola JK, Eppig JJ. 2003. A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence. *Biol Reprod*, 68:1682-1686.

Rose UM, Hanssen RGJM, Kloosterboer HJ. 1999. Development and characterization of an in vitro ovulation model using mouse ovarian follicles. *Biol Reprod*, 61:503-511.

Sebrell WH, Harris RS. 1967. *The Vitamins, Chemistry, Physiology, Pathology and Methods*. New York: Academic Press. v.1, pp. 305-320.

Sies H, Stahl W, Sundquist AR. 1992. Antioxidant functions of vitamins. *Ann N Y Acad Sci*, 669:7-20.

Tamilmani G, Rao BS, Vagdevi R, Amarnath D, Naik BR, Mutharao M, Rao VH. 2005. Nuclear maturation of ovine oocytes in cultured preantral follicles. *Small Rumin Res*, 60:295-305.

Tatemoto H, Ootaki K, Shigeta K, Muto N. 2001. Enhancement of developmental competence after in vitro fertilization of porcine oocytes by treatment with ascorbic acid 2-O-a-Glucoside during in vitro maturation. *Biol Reprod*, 65:1800-1806.

Thomas FH, Leask R, Srsen V, Riley SC, Spears N, Telfer EE. 2001. Effect of ascorbic acid on health and morphology of bovine preantral follicles during long-term culture. *J Reprod Fertil*, 122:487-495.

Tilly JL, Tilly KI. 1995. Inhibitors of oxidative stress mimic the ability of follicle-stimulating hormone to suppress apoptosis in cultured rat ovarian follicles. *Endocrinology*, 136:242-252.

Tsuji M, Ito Y, Terada N, Mori H. 1989. Ovarian aromatase action in scorbutic mutant rats unable to synthesise ascorbic acid. *Acta Endocrinol,* 121:595-602.

Woessner Jr JF. 1991. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J,* 5:2145-2154.

Wu D, Cheung QC, Wen L, Li J. 2006. A growth-maturation system that enhances the meiotic and developmental competence of porcine oocytes isolated from small follicles. *Biol Reprod*, 75:547-554.

Zreik TG, Kodaman PH, Jones EE, Olive DL, Behrman H. 1999. Identification and characterization of an ascorbic acid transporter in human granulosa–lutein cells. *Mol Hum Reprod,* 5:299-302.