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Essential role of follicle stimulating hormone in the maintenance of caprine preantral follicle viability *in vitro*

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Summary

The aims of the present study were to investigate the effects of follicle-stimulating hormone (FSH) on survival, activation and growth of caprine primordial follicles using histological and ultrastructural studies. Pieces of caprine ovarian cortex were cultured for 1 or 7 days in minimum essential medium (MEM – control medium) supplemented with different concentrations of FSH (0, 10, 50 or 100 ng/ml). Small fragments from non-cultured ovarian tissue and from those cultured for 1 or 7 days in a specific medium were processed for classical histology and transmission electron microscopy (TEM). Additionally, effects of FSH on oocyte and follicle diameter of cultured follicles were evaluated. The results showed that the lowest percentage of normal follicles was observed after 7 days of culture in control medium. After 1 day of culture, a higher percentage of growing follicles was observed in the medium supplemented with 50 ng/ml of FSH. In the presence of 10 and 50 ng/ml of FSH, an increase in diameter of both oocyte and follicle on day 7 of culture was observed. TEM showed ultrastructural integrity of follicles after 1 day of culture in MEM and after 7 days in MEM plus 50 ng/ml FSH, but did not confirm the integrity of those follicles cultured for 7 days in MEM. In conclusion, this study demonstrated that FSH at concentration of 50 ng/ml not only maintains the morphological integrity of 7 days cultured caprine preantral follicles, but also stimulate the activation of primordial follicles and the growth of activated follicles.

Keywords: Activation, Caprine, Culture, FSH, Primordial follicles

Introduction

Culture systems for primordial follicles are important for studying their oocyte development, especially because these follicles are a large potential source of oocytes that could be used *in vitro* for embryo production. The factors that control primordial follicle activation and further growth of primary follicles are not well understood. Since the cortical region where the primordial follicles are located is poorly vascularized, the development of these follicles is probably regulated by locally produced growth factors.

Endocrine hormones, like FSH, are known to regulate the production of several growth factors that play a critical role in primordial follicle activation and growth. FSH acts by binding to its receptor expressed on granulosa cells (Ulloa-Aguirre *et al.*, 1995; O'Shaughnessy *et al.*, 1996) and more recent reports indicate its presence in oocytes, suggesting additional sites of action in the ovary (Meduri *et al.*, 2002). Although FSH receptors are expressed from the primary follicles onward (Oktay *et al.*, 1997), FSH may play an indirect effect on very early follicle

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development via factors released by larger follicles or ovarian stromal cells. For example, FSH promotes proliferation of granulosa cells via paracrine factors such as IGF-1 and activin (van den Hurk & Zhao, 2005). In addition, FSH regulates expression of kit ligand (KL), growth differentiation factor-9 (GDF-9) and bone morphogenetic protein-15 (BMP-15) in murine follicles (Joyce *et al.*, 1999, Thomas *et al.*, 2005). These factors have been implicated in the activation of primordial follicles (van den Hurk & Zhao, 2005).

Studies *in vitro* have demonstrated that addition of FSH to culture media promotes preantral follicular growth and antrum formation in many species (mouse: Spears *et al.*, 1998; murine: McGee *et al.*, 1997; human: Wright *et al.*, 1999; bovine: Gutierrez *et al.*, 2000; ovine: Cecconi *et al.*, 1999; Zhou & Zhang, 2005; swine: Mao *et al.*, 2002). Moreover, it is known that FSH inhibits apoptosis in preantral follicles cultured *in vitro* (mouse: Baker *et al.*, 1997; rat: McGee *et al.*, 1997; human: Roy & Treacy, 1993). In goats, Silva *et al.* (2004) demonstrated that FSH, at concentration of 100 ng/ml, increased follicle and oocyte diameters after 5 days of culture, but no effect of FSH on both primordial to primary follicle transition and viability was observed. Clearly, FSH is known to be the main regulator of follicle development *in vivo* and *in vitro*, but a specific role or the possibility of dose-dependent action of FSH in promoting primordial to primary follicle transition and growth have not been tested, being essential to investigate whether FSH play a role in this step of folliculogenesis. In addition, most of the studies investigating primordial follicle activation are based on histological evaluation, being very important to use ultrastructural analysis to confirm follicular viability.

Studies with goats are important to improve our knowledge about the factors that control early folliculogenesis in mammals and to explore possible physiological differences among species. Goats are present on all continents and are commercially seen as highly attractive livestock, since they constitute an important source of products such as meat, milk, fibre and skin. Thus, the aim of the present study was to investigate whether FSH has a beneficial role in the survival, activation and further growth of *in vitro* cultured goat primordial follicles enclosed in ovarian cortex. To this end, both histological and ultrastructural studies were performed to investigate and compare the morphology of follicles before and after culture for 1 or 7 days in the absence or presence of FSH at different concentrations (0, 10, 50 or 100 ng/ml).

Materials and methods

Source of ovaries

Ovaries ($n = 10$) from five adult non-pregnant mixed-breed goats (1–3 years of age) were collected at a local slaughterhouse. The animals were cyclic and in good body condition. Then, the ovaries were washed and transported in 0.9% saline solution to the laboratory in thermo flasks with water at 32 °C.

Experimental protocol

At the laboratory, both ovaries from each animal were stripped of surrounded fat tissue and ligaments and cut in half, where after the medulla, large antral follicles and corpora lutea were removed. Following this, the ovarian cortex was divided into 11 fragments of approximately 3 × 3 mm (1 mm thick). One fragment was immediately fixed for classic histological studies (non-cultured controls) while a smaller fragment (1 mm^3) was randomly collected and subsequently fixed for ultrastructural examination. The other fragments of ovarian cortex were individually *in vitro* cultured in 1 ml of culture medium for 1 or 7 days at 39 °C with 5% CO_2 in air using a 24-well culture dish. The control medium was minimum essential medium (Cultilab, Brazil) supplemented with ITS (insulin 6.25 μg/ml, transferrin 6.25 μg/ml and selenium 6.25 ng/ml), 0.23 mM pyruvate; 2 mM glutamine; 2 mM hypoxanthine; 1.25 mg/ml BSA, 100 μg/ml penicillin, 100 μg/ml streptomycin (Vetec, Brazil) and 0.25 μg/ml fungizone (MEM^+). This control medium (MEM^+) was supplemented with different concentrations of porcine FSH (10, 50 or 100 ng/ml – provided by Dr J.F. Beckers, Liége, Belgium). All chemicals used in the present study were purchased from Sigma Chemical Co. unless otherwise indicated. Every 2 days, the culture medium was replaced by fresh medium. Each treatment was repeated five times, thus using the ovaries of five different animals.

Histological analysis and assessment of *in vitro* follicle growth

To evaluate the morphology of caprine follicles after 1 or 7 days of culture, a small part (1 mm^3) from each fragment was randomly removed for TEM studies, while the remainder was fixed in Carnoy for 12 h for histological studies. After fixation, the tissue fragments were dehydrated in a graded series of ethanol, clarified with xylene and embedded in paraffin wax. For each piece of ovarian cortex, 7 μm sections were mounted on slides, stained with periodic acid Schiff and hematoxylin (PAS staining system, Sigma) and examined by light microscopy (Zeiss) at ×100 and ×400 magnification.

The follicles were classified as described by Hulshof *et al.* (1994) in primordial (one layer of flattened granulosa cells around the oocyte) or growing follicles i.e. intermediate (one layer of flattened to cuboidal granulosa cells around the oocyte), primary (a single layer of cuboidal granulosa cells around the oocyte) or secondary (oocyte surrounded by two or more

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layers of cuboidal granulosa cells). These follicles were classified individually as histologically normal when an intact oocyte was present, i.e. an oocyte without a pyknotic nucleus, surrounded by granulosa cells which are well organized in one or more layers and that have no pyknotic nucleus. Degenerated follicles were defined as those with a retracted oocyte, which have a pyknotic nucleus and/or are surrounded by disorganized granulosa cells, which are detached from the basement membrane. From each medium and each culture period, approximately 150 follicles were randomly evaluated.

To evaluate follicular activation and growth, only intact follicles with a visible oocyte nucleus were recorded and the proportion of primordial and growing follicles were calculated at day 0 (control) and after 1 or 7 days of culture in the various media tested. Major and minor axes of each oocyte and follicle were measured with the aid of an ocular micrometer. The averages of the minor and major axes were reported as oocyte and follicle diameters, respectively. These values were used to assess the effect of the hormonal treatment on follicular growth.

Ultrastructural analysis

For ultrastructural analysis, small pieces of ovarian cortex were fixed in 2% paraformaldehyde, 2.5% glutaraldehyde and 0.1 M sodium cacodylate buffer, pH 7.2. After washing the ovarian pieces with sodium cacodylate buffer, they were postfixed in 1% osmium tetroxide, 0.8% potassium ferricyanide and 5 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer. Subsequently, samples were dehydrated in a graded series of acetone and embedded in Spurr's epoxy resin. Firstly, semi-thin sections (3 μm) were cut on an ultramicrotome (Reichert Supernova, German) for light microscopy studies and stained with toluidine blue. Subsequently, follicles classified as histologically normal in semi-thin toluidin blue-stained sections were submitted to ultrastructural analysis. For that purpose, thin sections (70 nm) were cut and then contrasted with uranyl acetate and lead citrate and examined using a Jeol 1011 (Jeol, Tokyo, Japan) transmission electron microscope, operating at 80 kV.

Statistical analysis

Data are expressed as mean ± SEM. The percentages of surviving follicles at all stages, primordial and growing follicles obtained after 1 or 7 days in the various treatments were subjected to arc–sin transformation before analysis of variance (ANOVA). The data as well as the diameter of oocytes and follicles were analysed by ANOVA followed by Fisher's protected least significant difference test (PLSD test) (StatView for Windows, SAS Institute Inc.). Values were considered statistically significant when $p < 0.05$.

Results

Effect of FSH and culture periods on follicle survival

Figure 1 shows the effect of different concentrations of FSH on follicle survival, i.e. the percentage of histologically normal follicles in ovarian tissues after 1 and 7 days of culture. When compared to non-cultured follicles, a significant decrease ($p < 0.05$) in the percentages of histologically normal follicles (Fig. 2) was observed after 1 and 7 days culture, but no significant effect of FSH on follicle survival was observed. With the increase of the culture period from 1 to 7 days, a significant ($p < 0.05$) decrease in the percentage of normal follicles was observed in control medium (MEM^+), but not in medium with FSH (Fig. 1).

Goat primordial follicle activation and growth during *in vitro* culture

The percentages of primordial and growing follicles in ovarian cortical tissue before and after 1 and 7 days of culture are shown in Table 1. The percentages of primordial and growing follicles in non-cultured cortex were 81.7 and 18.3%, respectively. After 1 day of culture, when compared to non-cultured follicles, a reduction ($p < 0.05$) of primordial follicles concomitant with a significant increase of growing follicles was observed in all treatments, except when 10 ng/ml FSH was

Table 1 Percentages (mean ± SEM) of primordial and growing follicles (intermediate, primary and secondary) in non-cultured tissues and in tissues cultured for 1 or 7 days in MEM^+ (control medium) and MEM^+ supplemented with various concentrations of FSH

	Primordial follicles	Growing follicles
Non-cultured (day 0)	81.7 ± 1.2^{a}	18.3 ± 1.2^{b}
Cultured (day 1)		
MEM^+	$71.7 \pm 8.7^{a,c,e}$	$28.3 \pm 4.6^{a,c,e}$
FSH 10	$81.5 \pm 2.7^{a,c}$	$18.5 \pm 3.7^{a,c}$
FSH 50	$45.3 \pm 4.7^{a,d,e}$	$54.7 \pm 6.2^{a,d,e}$
FSH 100	$62.2 \pm 2.7^{a,c,e}$	$37.8 \pm 2.9^{a,c-e}$
Cultured (day 7)		
MEM^+	$49.4 \pm 5.0^{b,c,e}$	$50.6 \pm 4.8^{a,c,e}$
FSH 10	$45.2 \pm 5.0^{b,c,e}$	$54.8 \pm 4.4^{b,c,e}$
FSH 50	$49.0 \pm 2.7^{a,c,e}$	$51.0 \pm 2.8^{a,c,e}$
FSH 100	$38.7 \pm 1.6^{b,c,e}$	$61.3 \pm 2.3^{b,c,e}$

Per treatment, 150 follicles were evaluated

[a,b] Different letters in the same column denote significant differences between culture periods within the same medium ($p < 0.05$).

[c,d] Different letters in the same column denote significant differences among treatments in the same period ($p < 0.05$).

[e] $p < 0.05$, significantly different from non-cultured ovarian cortex tissue (control/day 0).

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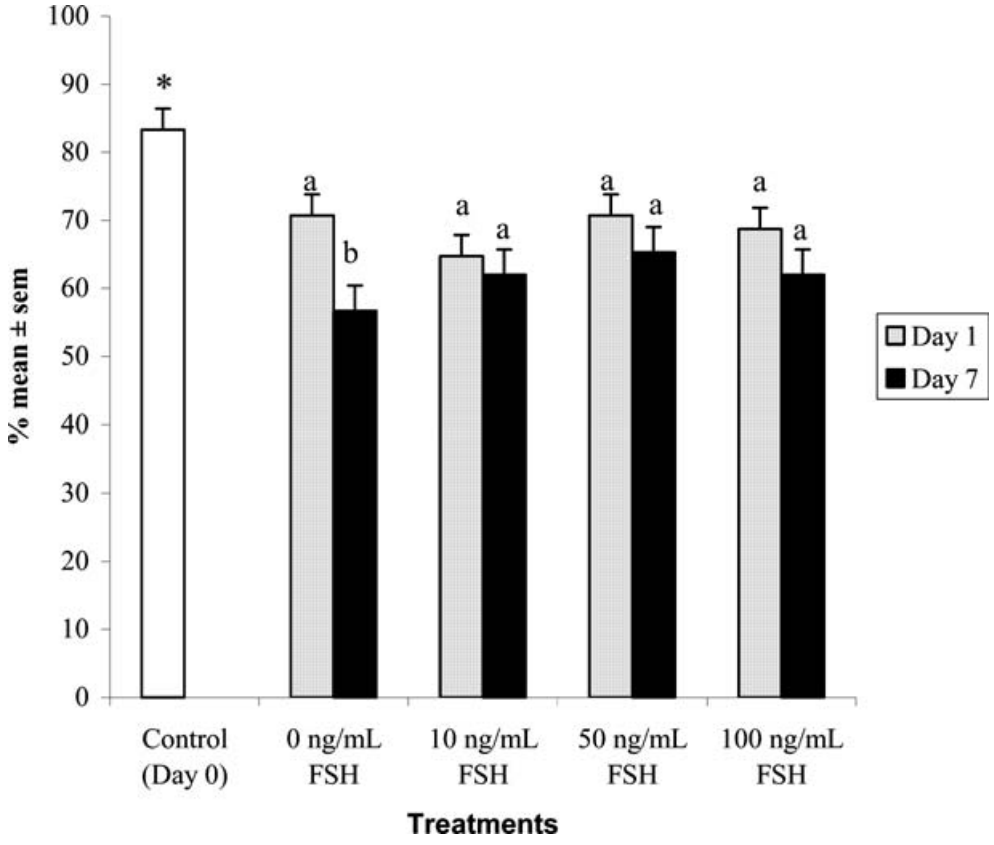

Figure 1 Percentages (means ± SEM) of histologically normal preantral follicles in non-cultured tissue (control) and in tissue cultured for 1 and 7 days in MEM$^+$ and MEM$^+$ supplemented with 10, 50 and 100 ng/ml FSH. (30 follicles evaluated in each one of five replicates per treatment). $^*p < 0.05$, significantly different from non-cultured ovarian cortex tissue (control/D0). (*a*, *b*) Different letters denote significant differences between culture periods within the same medium ($p < 0.05$).

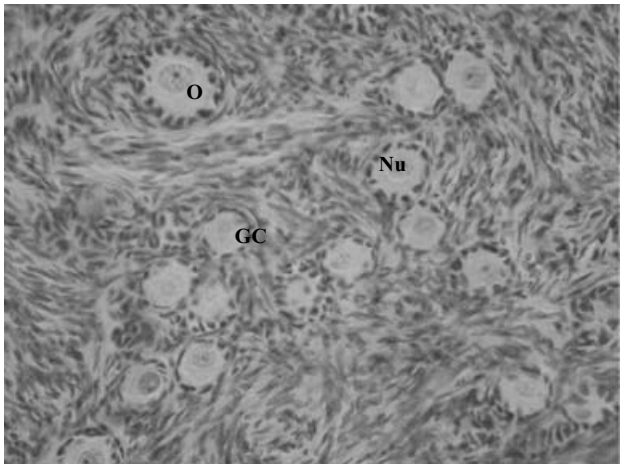

Figure 2 Histological section of non-cultured tissue after staining with periodic acid Schiff–hematoxylin, showing normal primordial and primary follicles. O: oocyte; NU: oocyte nucleus; GC: granulosa cells (×400).

added to the culture medium. Compared to day 1, after 7 days culture, a strong reduction of primordial follicles ($p < 0.05$) was observed, except in tissues cultured in MEM plus 50 ng/ml FSH. Cortical tissues cultured with 10 or 100 ng/ml of FSH had a significant increase ($p < 0.05$) in the percentage of growing follicles after 7 days of culture when compared to day 1. At day 1 of culture, addition of 50 ng/ml of FSH to control medium significantly decreased ($p < 0.05$) the percentage of primordial follicles when compared to non-cultured tissue and other treatments. Furthermore, addition of 50 ng/ml FSH increased significantly ($p < 0.05$) the percentage of growing follicles when compared to other treatments, except when 100 ng/ml was used.

After 7 days of culture in medium containing 10, 50 or 100 ng/ml of FSH, a significant increase in oocyte diameter was seen in unilaminar follicles, i.e., primordial, intermediate and primary follicles together, when compared to non-cultured tissue (Table 2; $p < 0.05$). In addition, the presence of 10 or 50 ng/ml of FSH promoted a significant ($p < 0.05$) increase in follicular diameter after 7 days of culture when compared to control. A significant increase in follicle diameter was observed with the increase of culture period from day 1 to 7 only in tissues cultured with 50 ng/ml of FSH. At day 7, tissue cultured in presence of 50 ng/ml FSH had the highest follicle diameter

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Table 2 Oocyte and follicle diameters (mean ± SEM) in non-cultured tissues and in tissues cultured for 1 or 7 days in MEM$^+$ (control medium) and MEM$^+$ supplemented with various concentrations of FSH. Per treatment 150 follicles were evaluated

	Oocyte diameter (μm)	Follicle diameter (μm)
Non-cultured (day 0)	40.7 ± 2.2	52.2 ± 2.2
Cultured (day 1)		
MEM$^+$	$38.6 \pm 1.9^{a,c}$	$51.6 \pm 2.1^{a,c}$
FSH 10	$40.9 \pm 2.3^{a,c}$	$53.1 \pm 2.6^{a,c}$
FSH 50	$39.7 \pm 2.3^{a,c}$	$54.4 \pm 2.9^{a,c}$
FSH 100	$36.9 \pm 2.3^{a,c}$	$51.0 \pm 2.5^{a,c}$
Cultured (day 7)		
MEM$^+$	$39.6 \pm 2.0^{a,c}$	$53.0 \pm 2.2^{a,c}$
FSH 10	$44.0 \pm 2.3^{a,c,e}$	$59.3 \pm 3.4^{a,c,e}$
FSH 50	$44.3 \pm 3.2^{a,c,e}$	$66.6 \pm 6.3^{b,d,e}$
FSH 100	$41.3 \pm 2.^{a,c,e}$	$54.9 \pm 2.3^{a,c}$

[a,b] Different letters in the same column denote significant differences between culture periods within the same medium ($p < 0.05$).
[c,d] Different letters in the same column denote significant differences among treatments in the same period ($p < 0.05$).
[e] $p < 0.05$, significantly different from non-cultured ovarian cortical tissues (control/Day 0).

($p < 0.05$). Cultured and non-cultured tissue contained variable and relatively low (often zero) numbers of secondary follicles, which were not amenable to statistical comparison.

Ultrastructural analysis of goat preantral follicles

Based on histological results, TEM studies were performed in non-cultured follicles (control) and in follicles cultured for 1 and 7 days in MEM$^+$ (control medium) or MEM$^+$ plus 50 ng/ml FSH. The ultrastructural characteristics of follicles from non-cultured tissue and those cultured in medium without or with 50 ng/ml FSH for 1 day appeared similar, but after 7 days only tissues cultured with FSH had normal follicles. These follicles exhibited sparse vesicles spread throughout the ooplasm. The homogeneous cytoplasm furthermore contained numerous rounded mitochondria with peripheral cristae and continuous mitochondrial membranes, although there were occasional elongated forms with parallel cristae (Fig. 3). Golgi complexes were rarely observed. Both smooth and rough endoplasmic reticulum were present, either as isolated aggregations or as complex associations with mitochondria and vesicles (Fig. 4). The oocyte nucleus had uncondensed chromatin and the nucleolus could generally be observed. In all developmental stages, granulosa cells were small, with a greater nuclear-to-cytoplasm ratio as compared with typical

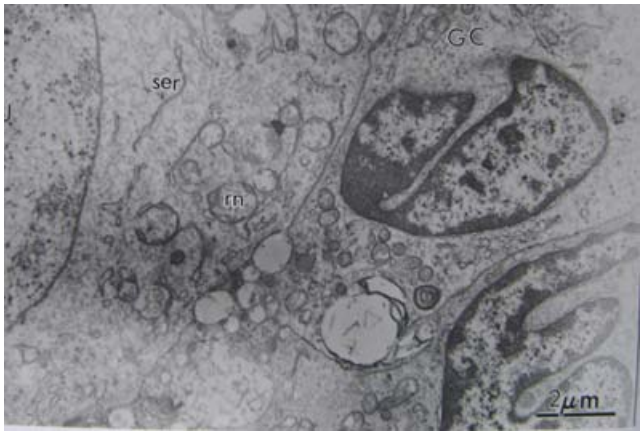

Figure 3 Electron micrograph of a preantral follicle from a non-cultured control ovarian fragment. Note the homogeneous cytoplasm with numerous rounded mitochondria. GC: granulosa cells; m: mitochondria; ser: smooth endoplasmic reticulum (×6600).

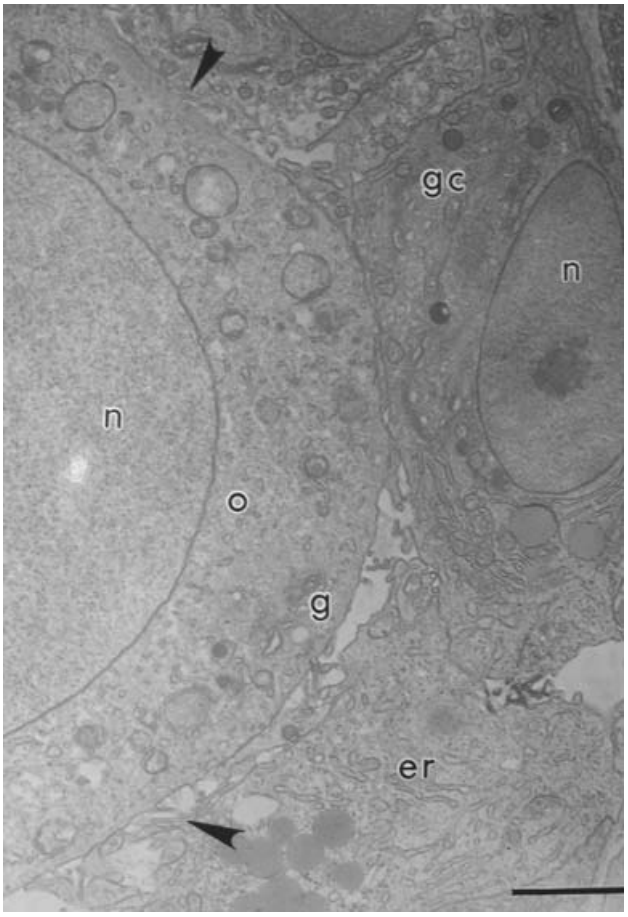

Figure 4 Electron micrograph of a preantral follicle cultured for 1 day in MEM$^+$ (cultured control). Note the great nuclear-to-cytoplasm ratio in granulosa cells. n: nucleus; o; oocyte; g: Golgi complex; gc: granulosa cells; er: endoplasmic reticulum; arrowhead: microvillus (×4000). Bar: 3 μm.

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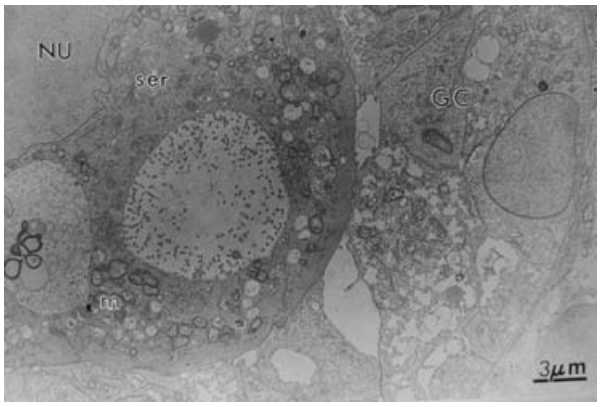

Figure 5 Electron micrograph of a preantral follicle cultured in FSH (50 ng/ml) for 7 days. Note the smooth endoplasmic reticulum and mitochondria, which were the most evident organelles observed in ooplasm. NU: oocyte nucleus; GC: granulosa cells; m: mitochondria; ser: smooth endoplasmic reticulum; v: vesicle (×3300).

cell structures. The nuclei were irregularly shaped, with loose chromatin in the inner part and small peripheral aggregates of condensed chromatin. Well-developed rough endoplasmic reticulum and mitochondria with well-developed lamellar cristae were the most evident organelles observed in granulosa cells. Gap junctions were abundantly observed between granulosa cells as well as between granulosa cells and the oocyte (Fig. 5).

Cortical tissues cultured in control medium for 7 days had histologically normal follicles, but TEM studies revealed some changes in their ultrastructure, which are indicative of degeneration. Such follicles showed an oocyte extremely vacuolated, with the vacuoles often being fused and producing a larger vacuolated area. Organelles were more randomly distributed throughout the cytoplasm and signs of endoplasmic reticulum proliferation and damage to mitochondrial membranes and cristae were observed. The oocyte nucleus appeared misshapen and retracted and had a wavy membrane. Granulosa cells look swollen, while the density of cytoplasmic organelles was low. Frequently, the connection between the oocyte and granulosa cells had disappeared, while organelles were no longer identifiable. Furthermore, granulosa cells showed less contact with each other and exhibited obvious fewer gap junctions (Fig. 6).

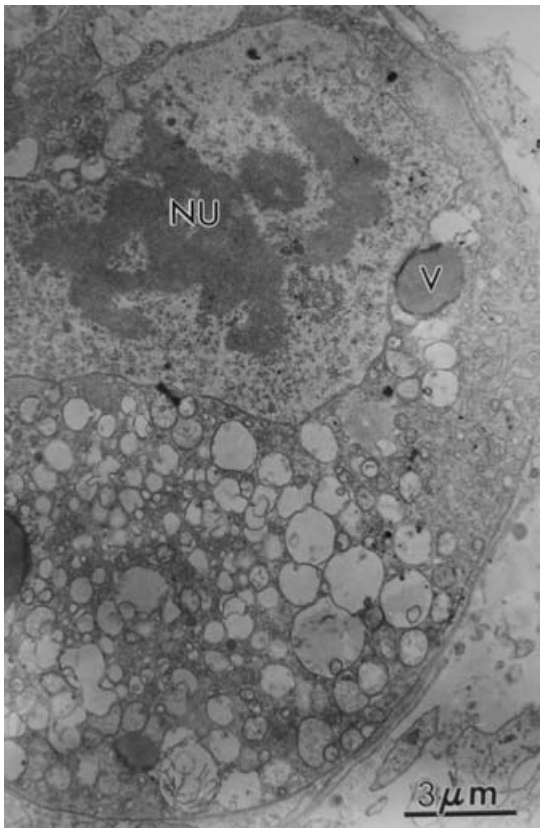

Figure 6 Electron micrograph of a preantral follicle cultured for 7 days in MEM^{+} (cultured control) that were scored as histologically normal at the LM level. Note the extreme vacuolization and the great holes present in the cytoplasm, indicative of degeneration. NU: oocyte nucleus; v: vesicles (×3900).

Discussion

The present study demonstrated the importance of FSH on *in vitro* activation and growth of caprine primordial follicles in a 7 days culture system. Although very little is known about the regulation of primordial follicle development, FSH seems to be a very effective factor in maintaining follicle viability (Ralph *et al.*, 1996; Wandji *et al.*, 1996; Saha *et al.*, 2000). However, Silva *et al.* (2004) did not observed a significant effect of FSH on follicle survival after 5 days culture, probably because a high concentration of FSH (100 ng/ml) was used. In our study, addition of 50 ng/ml of FSH to culture medium was very important to maintain the percentage of normal follicles after 7 days culture. Hsueh *et al.* (1994) and Chun *et al.* (1994) suggested that the diffusion of several essential chemical and physical factors through the basal membrane could be compromised in the absence of FSH. In addition, cultures without FSH more frequently resulted in extrusion of the oocyte from its original follicular structure (Cortvrindt *et al.*, 1997), which may be caused either by damage or reduction in number of gap junctions (Amsterdam & Rotmensch, 1987; Hsueh *et al.*; 1994). In addition, FSH inhibited apoptosis in preantral follicles cultured *in vitro* (mouse: Baker & Spears, 1997; rat: McGee *et al.*, 1997; human: Roy & Treacy, 1993; Wright *et al.*, 1999; swine: Mao *et al.*, 2002).

Addition of 50 ng/ml of FSH to the medium increased the activation rate of primordial follicles

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as early as day 1 of culture when compared to other treatments. However, after 7 days of culture, follicular activation was similar among all treatments. Ovarian follicular development is known to proceed to primordial and primary stages independently of the action of FSH. This has been observed in mice carrying invalidations of the FSHβ and FSHR genes (Kumar *et al.*, 1997; Dierich *et al.*, 1998) and also in patients with mutations suppressing the function of the FSHR (Beau *et al.*, 1998; Touraine *et al.*, 1999). FSH binds to its receptor expressed on granulosa cells (Ulloa-Aguirre *et al.*, 1995; O'Shaughnessy *et al.*, 1996) and oocytes (Meduri *et al.*, 2002) from the primary follicles onward (Oktay *et al.*, 1997). Recently, after culture of caprine ovarian cortical tissue for 5 days, Silva *et al.* (2004) showed that FSH (100 ng/ml) did not promote activation of caprine primordial follicles. Previous studies have confirmed that FSH at this concentration did not influence bovine follicular activation (Fortune *et al.*, 1998; Braw-Tal & Yossefi, 1997; Derrar *et al.*, 2000). Conversely, we demonstrated that a lower concentration of FSH (50 ng/ml) can improve follicular activation and survival. In addition, Joyce *et al.* (1999) reported that FSH stimulates Kit ligand mRNA expression in granulosa cells of preantral follicles. Kit ligand (KL) has been shown to be essential for oocyte growth (Eppig, 2001; Nilsson & Skinner, 2001) and primordial follicle activation (Parrot & Skinne, 1999). FSH can also modulate the levels of BMP-15 and GDF-9 in growing follicles (Thomas *et al.*, 2005) and these growth factors are essential for primordial and primary follicle development in mice (Dong *et al.*, 1996) and sheep (Galloway *et al.*, 2000).

In the current study, follicular diameter had the highest increase when cultured in presence of 50 ng/ml for 7 days. Itoh *et al.* (2002) also demonstrated that 50 ng/ml FSH increased both oocyte and follicular diameters in 13 days cultured bovine follicles. FSH receptor expression has been reported to develop progressively during the transition from primordial to primary to secondary follicle (Oktay *et al.*, 1997). The presence of FSH receptors in these early follicles presumably explains the effect of FSH on oocyte growth in preantral follicles. Since there are FSH receptors in oocytes, it is possible that FSH must act in both cell types to promote follicular growth and development (Méduri *et al.*, 2002). Furthermore, a two-way exchange may occur between the oocyte and granulosa cells and a direct action of FSH on oocytes produces compounds whose diffusion into the granulosa cells is necessary for their proliferation and maturation (Méduri *et al.*, 2002). Other authors observed that FSH promotes an increase in follicular diameter and proliferation of granulosa cells (rats: McGee *et al.*, 1997; mouse: Nayudu & Osborn, 1992; Cortvrindt *et al.*, 1996, 1997, 1998; bovine: Wandji *et al.*, 1996; Saha *et al.*, 2000; caprine: Silva *et al.*, 2004; ovine: Cecconi *et al.*, 1999; human: Roy & Treacy, 1993; Wright *et al.*, 1999). In addition, FSH was found to stimulate antrum formation and steroidogenesis in granulosa cells (Nayudu & Osborn, 1992; Boland *et al.*, 1993; Ralph *et al.*, 1995, 1996; Wandji *et al.*, 1996; Abir *et al.*, 1997; Gutierrez *et al.*, 2000; Wu *et al.*, 2000; Mao *et al.*, 2002; Mitchell *et al.*, 2002; Kreeger *et al.*, 2005). Adriaens *et al.* (2004) reported that omission of FSH during the early preantral stage tends to compromise a maximal oocyte developmental competence.

Several authors have emphasized the importance of TEM after *in vitro* culture of preantral follicles, since it gives close insight into the ultrastructural characteristics of follicles, allowing a better evaluation of their quality (Van den Hurk *et al.*, 1998; Zhao *et al.*, 2000; Salehnia *et al.*, 2002). In the present study, preantral follicles cultured for 1 day in MEM without or with FSH (50 ng/ml) respectively, appeared ultrastructurally normal, which confirmed the results obtained in the histological studies. However, after 7 days of culture, TEM studies revealed differences in ultrastructural quality of follicles cultured without or with FSH (50 ng/ml). Although they had comparable histological morphology, follicles cultured in the presence of FSH maintained their ultrastructural integrity, while those cultured without FSH showed various signs of initial degeneration after 7 days culture and exhibited more clear degenerative features, like ooplasm vacuolization. Cytoplasmic vacuoles are characteristic signs of degeneration in oocytes (Silva *et al.*, 2000), granulosa (Hay *et al.*, 1976) and cumulus cells (Assey *et al.*, 1994) and may represent endoplasmic reticulum swelling (Tassel & Kennedy, 1980) or altered mitochondrial structure (Fuku *et al.*, 1995). In goat preantral follicles, mitochondria showing extensive swelling and disappearance of their cristae and endoplasmic reticulum that have increased in volume, were previously indicated as the first signs of degeneration (Silva *et al.*, 2001).

In conclusion, this study with caprine follicles showed that a concentration of 50 ng/ml FSH is able to promote the activation of primordial follicles and growth of activated preantral follicles. Furthermore, these data support the vital role of FSH in maintaining healthy oocyte growth and follicular ultrastructure after 7 days culture. This culture system should be useful for studying the regulation of early follicular growth and development, especially because these follicles represent a large source of oocytes that could be used *in vitro* for embryo production.

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