Different Follicle-Stimulating Hormone (FSH) sources influence caprine preantral follicle viability and development *in vitro*

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Abstract

The aim of this study was to evaluate the effects of pituitary (pFSH) or recombinant (rFSH) FSH on the survival and growth of caprine preantral follicles. Caprine ovarian tissues were *in vitro* cultured for one or seven days in Minimum Essential Medium (MEM) alone or containing 10, 50, 100 and 1000 ng/ml of pFSH or rFSH. Control tissues (non-cultured) and those cultured were processed for histological and ultrastructural studies. In addition, follicular and oocyte diameter were analysed. After seven days of culture, only 50 ng/ml of rFSH maintained the percentage of normal follicles similar to control. Moreover, 10 ng/ml of pFSH and all the concentrations of rFSH promoted primordial follicles activation. In addition, the presence of 50 ng/ml of rFSH promoted the highest follicular diameter at day seven of culture. In conclusion, 50 ng/ml of rFSH maintained the ultrastructural integrity of caprine preantral follicles, promoted primordial follicles activation and further growth of cultured follicles.

Key words:
Caprine.
FSH.
Growth.
Preantral follicles.

Introduction

Reproductive techniques, as preantral follicles *in vitro* culture, are important to the achievement of a large amount of oocytes to be used in *in vitro* embryo production system. Some authors showed that it is possible to produce live offspring[1] or embryos of rat[2], porcine[3] and bubalines[4] through the culture of oocytes from preantral follicles. Although the factors that regulate early folliculogenesis are not yet completely established, it is well-known that paracrine and endocrine factors are involved in this phase. Among the endocrine factors, Follicle Stimulating Hormone (FSH) is a heterodimeric glycoprotein synthesized and secreted by the anterior pituitary gland. It is composed by two subunits, a and b, being the b-subunit that confers biological specificity to the hormone.[5] The oligosaccharides attached to the FSH molecule form a variety of isoforms and the acidic isoforms were found to have a reduced bioactivity compared with the less acidic ones.[6] Although FSH receptors are expressed in granulosa cells[7] from primary follicles stage onward[8], this hormone may act indirectly in the primordial follicles through paracrine factors secreted by larger follicles or stroma cells.

Some *in vitro* studies have demonstrated that the addition of FSH to the culture medium promotes the maintenance of viability and preantral follicles growth in caprine[9], as well as antrum formation in different species[10,11,12]. However, the effect of FSH in the *in vitro* follicular culture depends on differences among some factors, such as species, purity degree of commercial preparations of FSH and different *in vitro* culture systems. Furthermore, it was hypothesized that not only the quantity but also the source and the quality of FSH, in terms of isoforms and purity, plays an important role in the early

follicular phase.[6]

FSH can be extracted from (1) the urine of postmenopausal women, consisting in human menopausal gonadotropin (hMG), (2) pituitary extract of domestic animals, essentially swine (pFSH) and ovine (oFSH), followed by hormone purification, and (3) recombinant technology using Chinese hamster ovary cells.[13] Recombinant DNA technology for FSH production has been successfully used in the programs of controlled ovarian stimulation in human assisted reproduction.[14] Although its high cost, recombinant FSH (rFSH) is a more pure and homogeneous hormone[13], which could propitiate a higher efficacy in the results of follicular growth. Regarding pFSH, even after purification, its final product has a small percentage of contamination by others pituitary hormones, such as Luteinizing (LH) and Thyroid Stimulating Hormone (TSH).[15] Recently, authors demonstrated that rFSH is more efficient than urinary FSH in the growth of *in vitro* cultured murine preantral follicles.[16] Other study reported that rFSH increases oocyte maturation rates due to a better affinity to their receptors.[17] In addition, authors verified that, in the presence of rFSH, there was a larger cumulus expansion and a better embryos quality after maturation than using pFSH.[13] However, the effects of different commercial preparations of FSH on the survival, activation and growth of caprine preantral follicles are not known. Furthermore, for evaluation of the preantral follicles morphology most studies are based on histological evaluation, being important the use of ultrastructural analysis to confirm follicular integrity. Then, the present study aims to compare the effect of different sources (pituitary or recombinant) of FSH on the survival, activation and growth of preantral follicles enclosed in caprine ovarian cortex after *in vitro* culture.

Material and Method

Ovaries (n = 8) from four adult (1 – 3 years old), mixed-breed goats were obtained at a local slaughterhouse. Immediately after slaughter, the ovaries were removed, washed in 70% alcohol followed by two times in minimum essential medium (MEM) supplemented with 100 ìg/ml penicillin and 100 ìg/ml streptomycin. The material was transported in thermo flasks at 4 ºC to the laboratory within 1 hour.

Our organ culture system was described in detail earlier.[9] Ovarian tissue samples from each ovarian pair were cut in 21 fragments of approximately 3 × 3 mm (1 mm thick). One fragment (non-cultured control) was immediately fixed in Carnoy's fluid for 12 h for histological studies, while a smaller fragment (1 mm^3) was randomly collected and subsequently fixed in paraformaldehyde 2% and glutaraldehyde 2.5% in sodium cacodylate buffer 0.1 M (pH 7.2) for ultrastructural examination. The other fragments of ovarian cortices were individually *in vitro* cultured in 1 ml of culture medium for one or seven days at 39ºC with 5% CO_2 in air using a 24-well culture dish. The control medium was MEM supplemented with ITS (insulin 6.25 ìg/ml, transferrin 6.25 ìg/ml and selenium 6.25 ng/ml), 0.23 mM pyruvate; 2 mM glutamine; 2 mM hypoxanthine and 1.25 mg/ml BSA, called MEM^+. This control medium was tested alone (cultured control) or supplemented with different concentrations (10, 50, 100 or 1000 ng/ml) of pFSH (Folltropin®, Tecnopec, Brasil) or recombinant bovine FSH (rFSH®, Nanocore, Brasil). 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 and each treatment was repeated four times.

To evaluate the morphology of caprine follicles after one or seven days of culture, 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 ìm sections were mounted on slides, stained with periodic acid Schiff and hematoxylin and examined by light microscopy (Zeiss, Jena) at 100× and 400×

magnification.

The follicles were classified as described by Hulshof et al.[18] in primordial (one layer of flattened granulosa cells around the oocyte) and growing follicles i.e. primary (a single layer of cuboidal granulosa cells around the oocyte) or secondary (oocyte surrounded by two or more layers of cuboidal granulosa cells). 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 120 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 one or seven days of culture in the various media tested. To avoid counting a follicle more than once, preantral follicles were counted only when the oocyte nucleus was visible. Oocyte and follicle diameters before and after culture were analysed with the aid of an ocular micrometer.

For ultrastructural analysis, after fixation, the fragments were washed with sodium cacodylate buffer and postfixed in 1% osmium tetroxide, 0.8% potassium ferricyanide and 5 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer. Subsequently, samples were in bloc contrasted with uranyl acetate, dehydrated in a graded series of acetone and embedded in Spurr's epoxy resin. The follicles classified as histologically normal in the semi-thin sections stained with toluidin blue (3 mm) were submitted to ultrastructural analysis. For that purpose, ultra-thin sections (70 nm) were cut on an ultramicrotome (Reichert Supernova, German) and examined using a Jeol 1011 (Jeol, Tokyo, Japan) transmission electron microscope, operating at 80 kV.

Kolmogorov-Smirnov and Bartlett's tests were applied to confirm normal distribution and homogeneity of variance, respectively. Analysis of variance was made using GLM procedure of SAS (1999) and Dunnett's test was applied for comparison of control groups against each treatment tested. Student Newman Keuls' (SNK) test was used to compare percentages of surviving primordial or growing follicles among treatments and days of culture. Because of the higher coefficient of variation observed on follicles and oocytes diameters, Duncan's test was applied to compare treatments tested, whilst Student's t-test was used to compare means between days of culture. Differences among groups were considered significant when $p<0.05$ and results were expressed as means ± standard deviation (SD).

Results

A total of 2.280 caprine preantral follicles were analysed to verify follicular morphology. The percentage of viable follicles in non-cultured and cultured ovarian cortices is shown in figure 1. After one day of culture, it was observed a significant reduction ($P < 0.05$) in the percentage of normal follicles in treatments tested compared to control (80%), except when follicles were cultured in 10 and 50 ng/ml of rFSH ($P > 0.05$). In addition, after seven days of culture, the percentage of histologically normal follicles was similar ($P > 0.05$) to control only when 50 ng/mL of rFSH (70.83%) was used. Furthermore, only 10 and 50 ng/ml of rFSH showed percentages of normal follicles significantly higher ($P < 0.05$) than MEM (52.5%) at day seven. With the progression of the culture from one to seven days, there was a decrease ($P < 0.05$) in follicular viability in MEM^+ alone, pFSH 50 and 1000 ng/ml, as well as in rFSH 10 and 1000 ng/ml. Figure 2 presents normal caprine preantral follicles after seven days of culture with 50 ng/ml of rFSH, showing oocyte and granulosa cells integrity.

The percentages of primordial and growing follicles in non-cultured cortices were 83.3 and 16.7%, respectively (Figure 3). After one day of culture, a

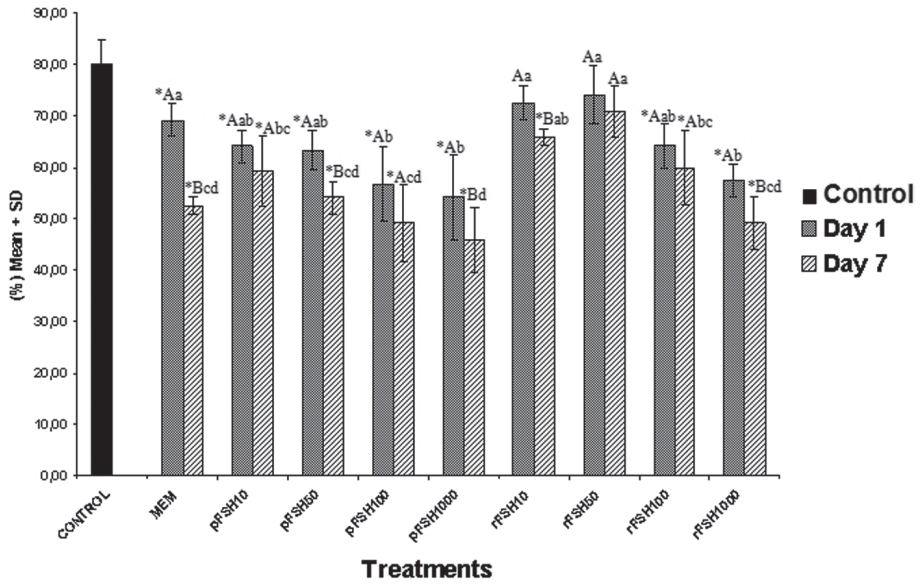

Figure 1 - Percentages (means ± SD) of histologically normal preantral follicles in non-culture tissue (control) and in tissue cultured for one or seven days in MEM⁺ and MEM⁺ supplemented with different concentrations of pFSH or rFSH. *p < 0,05, significantly different from non-cultured ovarian cortex tissue (control/D0). *A,B* Different letters in the same column denote significant differences between culture periods within the same medium ($p < 0.05$). (a, b, c, d) Different letters in the same column denote significant differences among treatments in the same period ($p < 0.05$)

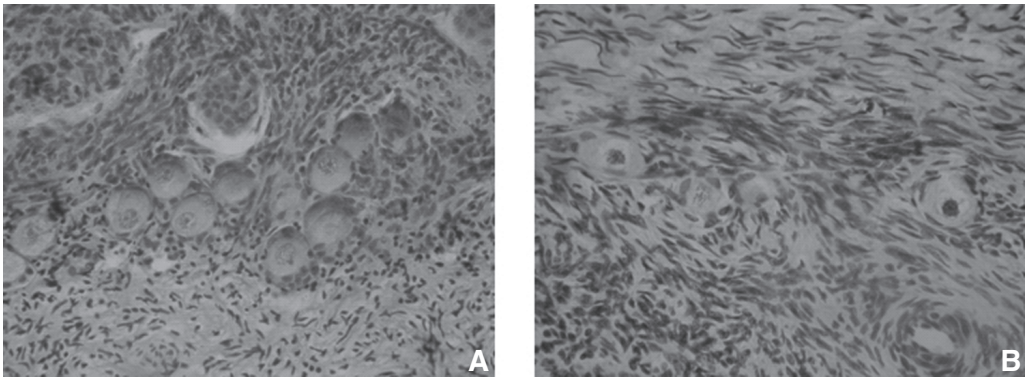

Figure 2 - Histological section of (A) culture tissue with rFSH 50 ng/ml (seven days of culture) after staining with periodic acid Schiff-hematoxylin, showing normal follicles and (B) culture tissue with pFSH 100 ng/ml, showing degenerate follicles. **o**: oocyty; **n**: oocyte nucleus; **gc**: granulose cells (x400)

significant reduction in the percentage of primordial follicles (Figure 3A; P < 0.05) concomitant with a significant increase in the percentage of growing follicles (Figure 3B; P < 0.05) were observed in all hormone-treated fragments when compared to control. On the other hand, it has not occurred to samples culture with MEM⁺ alone. Comparing results obtained after one and seven days of culture, MEM⁺ alone or the addition of 10 ng/ml of pFSH and all the concentrations of rFSH significantly decreased the percentage of primordial follicles (Figure 3A; P < 0.05) and increased the percentage of growing follicles (Figure 3B; P < 0.05). Furthermore, when comparisons were done among treatments at day seven, all rFSH concentrations had higher percentages of growing follicles (P < 0.05) than MEM⁺ alone.

Follicular and oocyte diameters are shown in table 1. After seven days of culture,

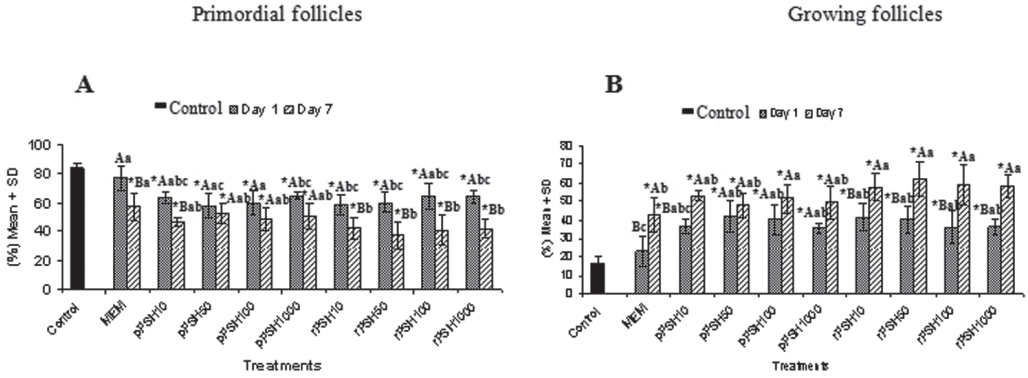

Figure 3 - Percentages (mean ± SD) of primordial (A) and growing follicles (primary and secondary) (B) in non-cultured tissues and in tissues cultured for one or seven days in MEM+ (control medium) and MEM+ supplemented with various concentrations of pFSH or rFSH. Per treatment, 120 follicles were evaluated. *p < 0,05, significantly different from non-cultured ovarian cortex tissue (control/D0). *A,B* Different letters in the same column denote significant differences between culture periods (one or seven days) within the same medium ($p < 0.05$). *a,b,c* Different letters in the same column denote significant differences among treatments in the same period ($p < 0.05$)

Table 1 - Oocyte and follicle diameters (mean ± SD) in non-cultured tissues and in tissues cultured for one or seven days in MEM+ (control medium) and MEM+ supplemented with various concentrations of FSH porcine and recombinant (n = 120)

	Oocyte diameter µm	Follicle diameter µm
Non-cultured (day 0)	55.00 ± 7.74	80.65 ± 11.73
Cultured (Day 1)		
MEM+	57.78 ± 4.38 Ac	86.21 ± 13.48 Aa
pFSH 10	64.58 ± 7.95*Aab	95.30 ± 14.47*Aa
pFSH 50	64.53 ± 8.45*Aab	93.01 ± 13.14*Aa
pFSH 100	66.71 ± 7.41*Aab	89.27 ± 14.10 Aa
pFSH 1000	68.17 ± 8.99*Aa	90.91 ± 14.10 Aa
rFSH 10	64.89 ± 8.14*Aab	93.51 ± 14.00*Aa
rFSH 50	67.98 ± 8.68*Aa	93.04 ± 12.71*Ba
rFSH 100	64.58 ± 8.96*Aab	89.45 ± 14.29 Aa
rFSH 1000	63.44 ± 8.26*Aab	91.93 ± 11.07*Aa
Cultured (day 7)		
MEM+	61.95 ± 7.86 Ab	93.51 ± 14.52*Ab
pFSH 10	59.79 ± 9.73 Ab	87.03 ± 12.77 Ab
pFSH 50	60.25 ± 8.36 Ab	86.84 ± 16.98 Ab
pFSH 100	61.18 ± 7.34 Bb	89.15 ± 15.89 Ab
pFSH 1000	69.06 ± 9.04*Aa	93.35 ± 13.05 Ab
rFSH 10	65.66 ± 8.56*Aab	92.70 ± 14.67 Ab
rFSH 50	69.28 ± 9.11*Aa	104.54 ± 16.63*Aa
rFSH 100	64.89 ± 8.45*Aab	93.63 ± 13.19*Ab
rFSH 1000	62.89 ± 8.67*Ab	92.01 ± 14.71 Ab

a,b,c Different letters in the same column denote significant differences among treatments in the same period ($p<0.05$). A,B Different letters in the same column denote significant differences between culture periods within the same medium ($p<0.05$). *p<0,05, significantly different from non-cultured ovarian cortex tissue (control/D0)

a significant increase ($P < 0.05$) in follicular diameter was seen in follicles cultured with MEM^+ alone or containing 50 or 100 ng/ml of rFSH when compared to non-cultured tissue (control). In addition, the presence of 50 ng/ml of rFSH promoted the highest follicular diameter ($P < 0.05$) at day seven of culture. Furthermore, only follicles cultured in the presence of 50 ng/ml rFSH increased follicular diameter from day one to seven ($P < 0.05$). With the increase of the culture period from day one to day seven, there was a significant decrease ($P<0.05$) in oocyte diameter after culture with 100 ng/ml of pFSH ($P < 0.05$). At day seven, oocyte diameters were significantly higher in 1000 ng/ml pFSH and 50 rFSH when compared to all other pFSH concentrations, 1000 ng/ml rFSH and MEM^+ alone.

Based on histological results, TEM studies were performed in non-cultured follicles (control, Figure 4A) and in follicles cultured for seven days in MEM^+ plus 50 ng/ml rFSH (Figure 4B). Follicles cultured for seven days in MEM^+ plus 50 ng/ml rFSH had ultrastructure very similar to control follicles. Both follicles showed intact basal and nuclear membranes, nucleus with descondensed chromatin, some vesicles and organelles uniformely distributed in the cytoplasm, with predominantly mitochondrias. The granulosa cells were normal, with alongated nucleus and a high proportion nucleus-cytoplasm. However, cortical tissues cultured with 50 ng/ml rFSH for seven days, showed granulosa cells discreetly detached from the oocyte.

Discussion

In this study, different sources of FSH (pituitary and recombinant) were compared to evaluate the influence of this hormone on caprine preantral follicles survival and growth *in vitro*. Although the two preparations are produced from different origins, they were tested in the same concentrations (10, 50, 100 or 1000 ng/ml). Our results demonstrated that, after seven days of culture, only rFSH at 50 ng/ml did not differ from control (non-cultured tissues) in relation to the percentage of normal follicles. On the other hand, in all concentrations tested, pFSH reduced the percentage of normal follicles after seven days. This result may be due to the fact that pFSH, a pituitary hormone, used in this study has trace contaminants of LH activity

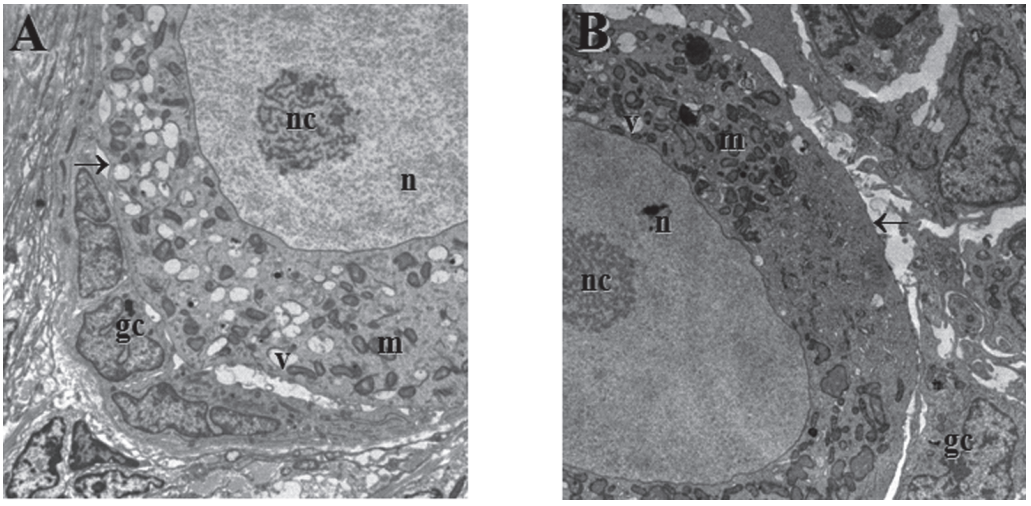

Figure 4 - Ultrastructural analysis of (A) a non cultured preantral follicle (5000X) and (B) a follicle cultured for seven days in medium containing 50 ng/ml rFSH (6000X). Note separation between granulosa cells and oocyte. **n**- nucleus, **gc**- granulosa cell, **nc**- nucleulus, **m**- mitochondria, **v**- vacuole, **arrow**- oocyte plasmatic membrane

(approximately 5.25:1) and rFSH is a pure preparation. One of the most important factors that alter hormone bioactivity is the purity degree after the preparation.[15] Thus, this experiment showed that the higher the FSH purity the better the efficiency of this hormone in the maintenance of preantral follicle viability after *in vitro* culture. According to some authors, LH alone or associated with FSH resulted in degeneration of caprine preantral follicles cultured *in vitro*.[19] Some authors also observed that rFSH[20,21] and pFSH[9] maintained viability and inhibit apoptosis of *in vitro* cultured preantral follicles in different species. Adversely to the benefic effects of FSH on follicular development, authors showed that pFSH induced degeneration in small bovine preantral follicles.[22] These contradictions may be due to differences between species, hormone sources and concentrations, as well as experimental design used in the culture.

In our study, addition of any concentration of rFSH, as well as 10 ng/ml of pFSH, increased the primordial follicle activation rates from day one to seven of culture. It is well-known that FSH is essential for normal follicular development until the preovulatory stages as well as for inducing its own receptors and LH receptors in granulosa cells.[23] Authors reported that FSH stimulates the expression of some growth factors[24] such as Kit Ligand[25], which is important for the regulation of early folliculogenesis. In the present study, the best results of activation obtained with rFSH may be due to the hormone purity, providing a higher efficacy. Moreover, evidences showed that different isoforms have differential capability to bind to target-cell receptors and to evoke biological responses.[26] Thus, rFSH has less acidic isoforms, which bind to FSH receptors with a higher affinity, resulting in the increase in hormone bioactivity.[16] Other studies demonstrated that rFSH, when compared to urinary FSH, induces better proliferation of granulosa cells[16] as well as estradiol production[27].

In the present study, the highest increase in follicular diameter was obtained when the follicles were cultured in the presence of 50 ng/ml of rFSH. Others authors also demonstrated that rFSH increased bovine follicular and oocyte diameters after culture for thirteen days[28]. In addition, others authors reported that the expression of FSH receptors developed progressively during the transition from primordial to primary and secondary follicles.[8] The presence of FSH receptors in granulosa cells suggests that FSH can promote follicular development and growth. Furthermore, the results from this study are important to assisted reproduction programs since FSH has been extensively used in female infertility treatment. Although its high cost, clinicians and couples may still prefer to use rFSH for reasons such as improvement in follicular growth and viability. On the other hand, in our study, pFSH (100 ng/ml) promoted a reduction in follicular diameter from day one to seven of culture. We suggest that this occurs due to oocyte degeneration from large preantral follicle during the culture period, since in the preantral phase, the oocyte is more sensitive to degeneration than granulosa cells.[29]

Transmission electron microscopy was used as a qualitative and supplementary technique to evaluate follicular integrity after *in vitro* culture. In the present study, preantral follicles of non-cultured control and those cultured for seven days with rFSH 50 ng/ml appeared ultrastructurally normal, which confirmed the results obtained in the histological studies, although cortical tissues cultured with 50 ng/ml rFSH for seven days showed granulosa cells separated from the oocyte. This find was also observed in porcine preantral follicle cultured *in vitro* (Lucci, personal communication), and it does not necessarily indicate follicular degeneration.

In conclusion, this study showed that 50 ng/ml of rFSH maintained the ultrastructural integrity of caprine preantral follicles and promoted primordial follicles activation and further growth of follicles cultured for seven days

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Diferentes origens do Hormônio Folículo Estimulante (FSH) influenciam a viabilidade e o desenvolvimento de folículos pré-antrais caprinos

Resumo

O objetivo deste estudo foi avaliar os efeitos do FSH pituitário (pFSH) ou recombinante (rFSH) sobre a sobrevivência e o crescimento de folículos pré-antrais caprinos. O tecido ovariano foi cultivado *in vitro* por um ou sete dias em Meio Essencial Mínimo (MEM) sozinho, ou contendo 10, 50, 100 e 1000 ng/ml de pFSH ou rFSH. O grupo controle (não cultivado) e aqueles cultivados foram processados para análises histológica e ultra-estrutural. Além disso, os diâmetros folicular e oocitário foram avaliados. Após sete dias de cultivo, apenas 50 ng/ml de rFSH manteve o percentual de folículos normais semelhante ao controle. Além disso, 10 ng/ml de pFSH e todas as concentrações de rFSH promoveram ativação de folículos primordiais. A presença de 50 ng/ml de rFSH promoveu o maior diâmetro folicular após sete dias de cultivo. Em conclusão, 50 ng/ml de rFSH manteve a integridade de folículos pré-antrais caprinos e promoveu a ativação e o crescimento dos folículos cultivados.

Palavras-chaves:
Caprinos.
FSH.
Crescimento folicular.
Folículos pré-antrais.

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