

## Impact of pituitary FSH purification on *in vitro* early folliculogenesis in goats

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**Key words:** goat, preantral follicles, FSH, follicular growth

**ABSTRACT:** Porcine pituitary follicle stimulating hormone (pFSH) is known to regulate the production of growth factors that have an essential role in early folliculogenesis. However, the effects of different preparations of pFSH on the survival and development of caprine follicles are not yet known. The aim of this study was to evaluate the effects of different pFSH (Stimufol<sup>®</sup> and Folltropin<sup>®</sup>) on the *in vitro* survival and growth of caprine preantral follicles. Pieces of caprine ovarian tissues were cultured for either one or seven days in a supplemented Minimum Essential Medium, alone or containing either Stimufol<sup>®</sup> (50 ng/mL) or Folltropin<sup>®</sup> (10, 50, 100 and 1000 ng/mL). Fresh control ovarian tissues as well as cultured tissues were processed for histological and ultrastructural studies. The results showed that after seven days, only Stimufol<sup>®</sup> maintained follicular morphology similar to control. Moreover, follicular degeneration was higher in medium alone or with Folltropin<sup>®</sup> at 50, 100 and 1000 ng/mL. However, at day seven, the percentage of growing follicles was higher in 100 ng/mL of Folltropin<sup>®</sup> than Stimufol<sup>®</sup>. In conclusion, FSH preparations affect differently the performance of *in vitro* culture of caprine preantral follicles. Stimufol<sup>®</sup> was better to preserve follicular morphology while Folltropin<sup>®</sup> was more efficient to promote follicular growth.

### Introduction

Over recent years, several researches have been done on the factors regulating early folliculogenesis, particularly in monovular species, such as domestic ruminants (Matos *et al.*, 2007; Saraiva *et al.*, 2008). These researches have raised the possibility that the store of oocytes enclosed in primordial follicles could be exploited to increase the reproductive potential of economically important animals, to preserve endangered species, as well as to treat infertility in clinical medi-

cine. In addition, the *in vitro* culture of preantral follicles allows the evaluation of the effects of different substances (hormones, growth factors, antibiotics, etc.) before their *in vivo* use, either in animals or humans.

Follicle-stimulating hormone (FSH) is a heterodimeric glycoprotein synthesized and secreted by the anterior pituitary gland under hypothalamic control, and which is involved in early folliculogenesis. FSH receptors are expressed in granulosa cells (Ulloa-Aguirre *et al.*, 2003; O'Shaughnessy *et al.*, 1996) from the primary follicle stage onward (Oktay *et al.*, 1997). It is well known that FSH can indirectly act in the primordial follicles through paracrine factors secreted by follicular or stromal cells, such as KIT ligand (Fiona *et al.*, 2005; Eppig, 2001). Some *in vitro* studies demonstrated that the addition of FSH to the culture medium promotes the inhibition of apoptosis and antrum for-

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mation in large isolated secondary follicles from different species (murine: McGee *et al.*, 1997; human: Wright *et al.*, 1999; ovine: Cecconi *et al.*, 1999; bovine: Gutierrez *et al.*, 2000; swine: Mao *et al.*, 2002). In spite of the beneficial effects of FSH on the *in vitro* follicular development, Nuttinck *et al.* (1996) showed that pFSH (0.43 pg FSH/pg proteins) induces degeneration in small bovine preantral follicles. The differences among these studies may, in part, due to the degree of purity of commercial preparations of FSH.

FSH can be obtained by pituitary extract of domestic animals, mainly swine and sheep, followed by hormone purification (Calder *et al.*, 2003). Even after its purification, porcine FSH (pFSH) has some contamination with other pituitary hormones, such as luteinizing hormone (LH) and thyroid stimulating hormone (TSH) (Closset and Hennen, 1989). There are two well known commercial preparations of pFSH, i.e.; Folltropin® (Bényei and Barros, 1999) and Stimufol® (Mazouz *et al.*, 1996). The FSH:LH ratio in Stimufol® is 20:1 (Mazouz *et al.*, 1996) and it is approximately 5.25:1 in Folltropin® (manufacturer's specifications, 2008). Both Folltropin® and Stimufol® have shown satisfactory results in the superovulation and embryo transfer protocols in different species (ovine: Cognie, 1999; bovine: Bényei and Barros, 1999; caprine: Baldassarre and Karatzas, 2004; Bonnet-Garnier, 2008). However, the effects of different commercial preparations of FSH on the survival, as well as on the *in vitro* development of caprine preantral follicles have not been studied.

In the current study we wanted to compare the effects of Stimufol® and Folltropin® on the viability (through both structural and ultrastructural analyses), activation of primordial follicles and further follicular growth on cultured caprine ovarian tissue.

## Materials and Methods

### *Source and preparation of ovarian tissue*

Ovaries (n = 8) from 4 adult (1–3 years old), mixed-bred goats (*Capra hircus*) were obtained at a local slaughterhouse. Immediately after slaughter, the ovaries were removed, washed once in 70% alcohol and twice in Eagle's minimum essential medium (MEM) supplemented with 100 µg/mL penicillin and 100 µg/mL streptomycin. The material was transported in thermo flasks at 4°C to the laboratory, and were submitted within 1 hour to the experimental procedures (as described below).

### *Caprine ovarian tissue culture*

At the laboratory, both ovaries from each animal were stripped of surrounding fat tissue and ligaments and cut in half. Then the ovarian cortex was isolated by removing the ovarian medulla, large antral follicles and corpora lutea. Afterwards, the ovarian cortex was divided into 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<sup>3</sup>) was 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 (see below) for either one or seven days at 39°C with 5% CO<sub>2</sub> in air using a 24-well culture dish. The culture medium used was a supplemented MEM (with 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 and 1.25 mg/mL bovine serum albumin). The control medium was tested alone (cultured control) or after adding either 50 ng/mL Stimufol® (provided by Dr J.F. Beckers, Liège, Belgium) or Folltropin (Tecnopoc, Brasil) in different concentrations (10, 50, 100 or 1000 ng/mL). 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. The concentration of 50 ng/ml of Stimufol® was chosen because it showed better results when compared to the other concentrations (1, 10 and 100 ng/ml) in previous experiments performed in our laboratory (Matos *et al.*, 2007).

### *Histological examination and assessment of in vitro follicle growth*

To evaluate the morphology of caprine follicles after one or seven days of culture, the fixed tissue fragments were dehydrated in a graded ethanol series, clarified with xylene and embedded in paraffin wax. Seven µm sections were mounted on slides, stained with periodic acid Schiff and hematoxylin, and examined by light microscopy at 100× and 400× magnification.

Follicles were classified as described by Hulshof *et al.* (1994) into primordial (one layer of flattened granulosa cells around the oocyte) and growing follicles. The latter were classified as "primary" (a single layer of cuboidal granulosa cells around the oocyte) or "secondary" follicles (oocyte surrounded by two or more

layers of cuboidal granulosa cells). Non viable follicles were defined as those in which the oocyte was shrunk, had a pyknotic nucleus and/or was surrounded by disorganized granulosa cells (detached from the basement membrane). 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 measured with the aid of an ocular micrometer. At least 120 follicles were randomly evaluated in each case.

### Ultrastructural analyses

After fixation, the fragments were washed with sodium cacodylate buffer and postfixed in 1% osmium tetroxide, 0.8% potassium ferricyanide and 5 mM CaCl<sub>2</sub> in 0.1 M sodium cacodylate buffer. Subsequently, samples were contrasted with uranyl acetate, dehydrated in a graded series of acetone and embedded in Spurr's epoxy resin. Follicles which were classified as histologically viable in toluidin blue-stained semi-thin sections (3 µm) 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.

### Statistical analyses

Kolmogorov-Smirnov and Bartlett's methods were applied to test normal distribution and homogeneity of variance, respectively. Analysis of variance was made

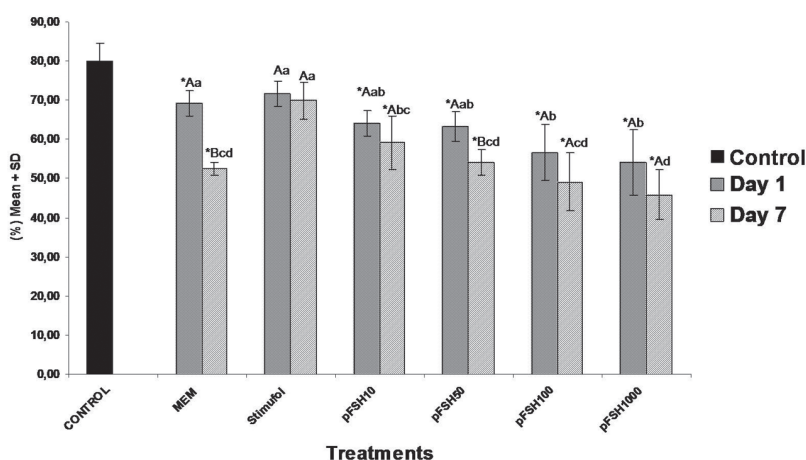
using General Linear Models (GLM) procedure of Statistical Analysis System (SAS, 1999) and Dunnett's test was applied for comparison of control groups against each treatment tested. Student Newman Keuls' test was used to compare percentages of surviving primordial or growing follicles among treatments and days of culture. Because of the higher coefficient of variation observed in both follicle and oocyte 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 between groups were considered significant when  $p < 0.05$  and results were expressed as mean  $\pm$  standard deviation (SD).

## Results

### Effect of FSH and culture duration on follicular viability

The percentage of normal preantral follicles in both cultured and non-cultured ovarian cortex is shown in figure 1. A total of 1560 preantral follicles were analysed to verify follicular morphology.

After one or seven days of culture, a significant reduction ( $P < 0.05$ , Dunnett test) in the percentage of viable follicles was observed in the cultured control as well as in all Folltropin® treatments when compared to the non-cultured control. However, the percentage of viable follicles in Stimufol® treated samples (50 ng/mL), did not differ from the non-cultured controls ( $P > 0.05$ , Dunnett test). Moreover, when comparisons were done among treatments, after seven days of culture, the highest percentage of viable follicles ( $P > 0.05$ , SNK test) was observed with 50 ng/mL of Stimufol®. In addition to that, Folltropin® appeared to promote a dose dependent reduction in the number of viable follicles cultured samples exposed to 1000 ng/mL of Folltropin® showed



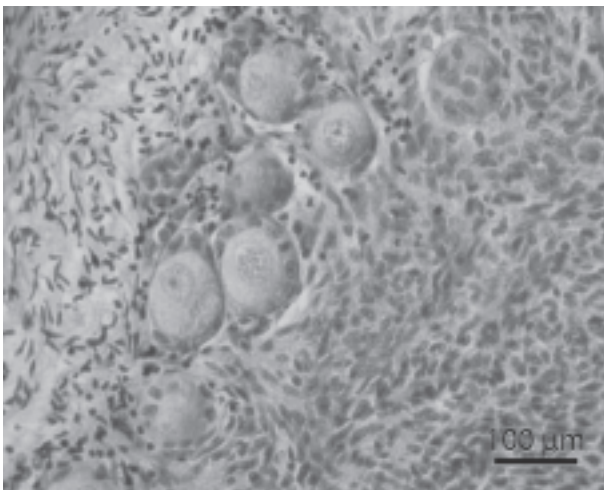
**FIGURE 1.** Percentages (means  $\pm$  SD) of histologically normal preantral follicles in non-cultured tissue (control) and in tissue cultured for one or seven days in medium with or without gonadotropin (either 50 ng/mL of Stimufol® or different concentrations of Folltropin®). \* $p < 0.05$ , significantly different from non-cultured ovarian cortex tissue (control/D0). A, B Different letters on the same column denote significant differences between culture periods within the same dose ( $p < 0.05$ ). (a, b, c, d) Different letters on the same column denote significant differences between treatments in the same period ( $p < 0.05$ ).

a significantly lower percentage of viable follicles than those found in 10 ng/mL treated samples ( $P < 0.05$ , SNK test). Also, after seven days of culture, there was a reduction ( $P < 0.05$ , SNK test) in the percentage of morphologically viable follicles in medium added with 50 ng/mL of Folltropin®, while all other Folltropin® treated groups did not differ significantly ( $P > 0.05$ , SNK test) in the percentage of viable follicles from the cultured control.

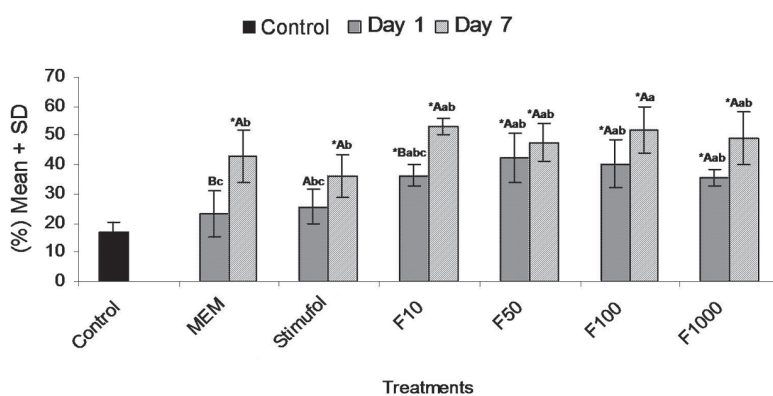
Figure 2 shows the morphological integrity of oocytes and granulosa cells in caprine preantral follicles after seven days of culture with 50 ng/mL of Stimufol®.

#### Follicle activation during *in vitro* culture

The percentage of growing follicles in non-cultured and cultured control tissue was 16.7% and 23.1%, respectively, and the difference was not statistically significant ( $P > 0.05$ , Dunnett test, Fig. 3).



**FIGURE 2.** Histological section of culture tissue with Stimufol® 50 ng/ml (seven days of culture) after staining with periodic acid Schiff-hematoxylin, showing normal follicles. **o**: oocyte **n**: oocyte nucleus; **gc**: granulosa cells



**FIGURE 3.** Percentages of growing follicles in non-cultured tissues and in tissues cultured for one or seven days with or without gonadotropins addition (either 50 ng/mL of Stimufol® or different concentrations of Folltropin®). About 120 follicles were evaluated per treatment. \* $p < 0.05$ , significantly different from non-cultured ovarian cortex tissue (non-cultured control and cultured tissue without gonadotropins). A,B Different letters on the same column denote significant differences between culture periods (1 vs 7 days) within the same dose ( $p < 0.05$ ; SNK test). a,b,c Different letters in the same column denote significant differences between treatments in the same period ( $p < 0.05$ ; SNK test).

After one day of culture, there was a significant increase in the percentage of growing follicles (Fig. 3;  $P < 0.05$ , SNK test) in fragments treated with Folltropin® when compared to the cultured control. With the progression of the culture to seven days, an increase in the percentage of growing follicles (Fig. 3B;  $P < 0.05$ , SNK test) was observed when the follicles were cultured in either medium alone or with 10 ng/mL of Folltropin®. In addition, when we compared the seven days treatments, tissues cultured with 100 ng/mL of Folltropin® showed a higher percentage of growing follicles ( $P < 0.05$ , SNK test) as compared with medium alone or with 50 ng/mL of Stimufol®.

#### Preantral follicle growth during *in vitro* culture

Follicular and oocyte diameters before and after the different culturing conditions are shown in Table 1. After seven days, a significant increase ( $P < 0.05$ , Student's *t* test) in follicular diameter was observed only in follicles cultured with medium alone when compared to non-cultured tissue (control), however, there was not difference in follicular diameters among treatments. With the increase of the culture period from day one to day seven, there was a significant reduction ( $P < 0.05$ , Student's *t* test) in oocyte diameter after using of 100 ng/mL of Folltropin® ( $P < 0.05$ , Student's *t* test). At day seven, oocyte diameter was significantly higher after culture with 1000 ng/mL of Folltropin® when compared with all other treatments.

#### Ultrastructural analysis of caprine preantral follicles cultured *in vitro*

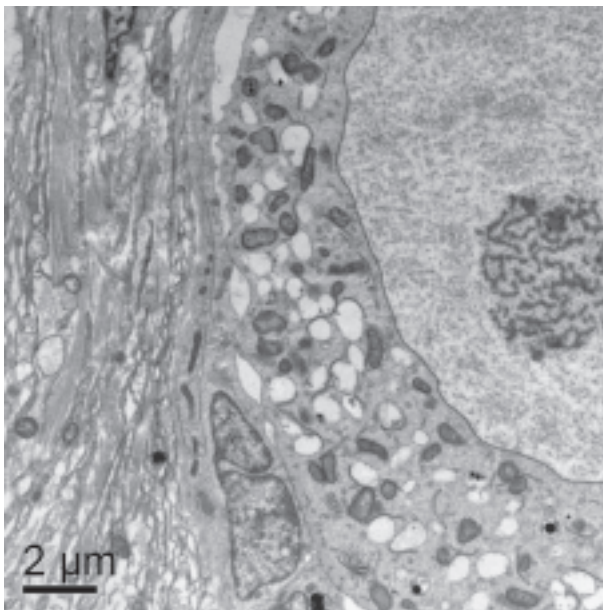
Based on histological results, transmission electron microscopy was performed in non-cultured follicles (control, Fig. 4A) and in follicles cultured for 7 days in MEM<sup>+</sup> with 50 ng/mL of Stimufol® (Fig. 4B), which

TABLE 1.

Oocyte and follicle diameters (mean±SD) in non-cultured tissues and in tissues cultured for either one or seven days in control medium or after the addition of 50 ng/mL of Stimufol® and various concentrations of Folltropin® (ng/mL). Number of cases was 120 per group.

	Oocyte diameter µm	Follicle diameter µm
Non-cultured (day 0)	55.00 ± 7.74	80.65 ± 11.73
One day cultures		
Medium alone	57.78 ± 4.38 Ac	86.21 ± 13.48 Aa
Stimufol® 50	61.34 ± 4.94 Abc	94.55 ± 6.60*Aa
Folltropin® 10	64.58 ± 7.95*Aab	95.30 ± 14.47*Aa
50	64.53 ± 8.45*Aab	93.01 ± 13.14*Aa
100	66.71 ± 7.41*Aab	89.27 ± 14.10 Aa
1000	68.17 ± 8.99*Aa	90.91 ± 14.10 Aa
Seven days cultures		
Medium alone	61.95 ± 7.86 Ab	93.51 ± 14.52*Ab
Stimufol® 50	61.80 ± 8.80 Ab	90.98 ± 12.32Ab
Folltropin® 10	59.79 ± 9.73 Ab	87.03 ± 12.77 Ab
50	60.25 ± 8.36 Ab	86.84 ± 16.98 Ab
100	61.18 ± 7.34 Bb	89.15 ± 15.89 Ab
1000	69.06 ± 9.04*Aa	93.35 ± 13.05 Ab

a.b.c Different letters in the same column denote significant differences between 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).



**FIGURE 4.** Ultrastructural images of (A) a non cultured preantral follicle (8000X) and (B) a follicle cultured for seven days in medium containing 50 ng/ml Stimufol (6000X). Note separation between granulosa cells and oocyte. **n**- nucleus, **gc**- granulosa cell, **nc**- nucleolus, **m**- mitochondria, **v**- vacuole, **arrow**- oocyte plasma membrane.

showed an ultrastructure similar to control. In both treatments, the follicle had intact basal and nuclear membranes, nucleus with descondensed chromatin and some vesicles. The organelles are uniformly distributed through the ooplasm, predominantly mitochondrias. Granulosa cells were ultrastructurally normal with an elongated nucleus and a high proportion nucleus-cytoplasm. It is important to note that we observed a slight detachment of the granulosa cells from the oocyte in follicles cultured in 50 ng/mL de Stimufol®.

## Discussion

One of the main factors that alter hormone bioactivity is the purity degree after its preparation (Closset and Hennen, 1989). In this study, two preparations of porcine pituitary FSH were evaluated on the viability and growth of caprine preantral follicles *in vitro*. Our results demonstrated that, after seven days, only the fragments of ovarian cortex cultured with Stimufol® had similar percentage of viable follicles when compared to control. On the other hand, all concentrations of

Folltropin reduced the percentage of morphologically viable follicles after seven days. This result can be due to the fact that Stimufol® did not have significant amounts of LH activity contaminants. Although they are both porcine preparations, the proportion of contamination by LH (FSH:LH) is 20:1 in Stimufol® (Mazouz *et al.*, 1996) and about 5,25:1 in Folltropin® (manufacturer's information). This difference in the FSH purity may be due to differences in the hormonal purification technique between each laboratory. The same procedure for separation or purification of FSH can result in changes in the isoforms of this hormone (Bousfield *et al.*, 2008). This suggests that the isoforms are influenced by the presence or absence of other proteins, such as LH. The current study showed that as higher the purity degree of FSH, as better the effectiveness of the preparation for the maintenance of caprine preantral follicles viability in an *in vitro* culture system. In another study, LH alone or in association with FSH resulted in degeneration of caprine preantral follicles cultured *in vitro* (Saraiva *et al.*, 2008). Some authors have shown that Stimufol® (Tsai-Turton and Luderer, 2006; Matos *et al.*, 2007) maintain viability and inhibit apoptosis of preantral follicles after *in vitro* culture in different species. Notwithstanding the beneficial effects of FSH on the *in vitro* follicular development, Nuttinck *et al.* (1996) have shown that pFSH (0.43 pg FSH/pg proteins) induces degeneration in small isolated bovine preantral follicles. These contradictions are likely due to the species, purity and hormone concentration differences, as well as in culture methodologies.

In the present study, we verify that addition of 10 ng/mL of Folltropin®, although did not provide high follicular viability rates, was capable to provide satisfactory activation rates in relation to Stimufol from day one to seven of culture. The inconstant ratio between FSH/LH in the commercial preparations of pFSH used, in part, can explain the results obtained in this study. It is known that FSH acts in early folliculogenesis (Joyce *et al.*, 1999; Nilsson and Skinner, 2001) and is essential to an adequate development up to the preovulatory stage, however, the role of LH in this phase is not clearly defined (Salveti *et al.*, 2007). Studies have demonstrated that Folltropin® provides satisfactory results in the superovulation and embryo transfer protocols in different species (ovine: Cognie, 1999; caprine: Baldassarre and Karatzas, 2004; bovine: Bényei and Barros, 1999). On the other hand, Beckers (1987) showed that the reduction in LH content in the commercial preparations of gonadotrophin (FSH/LH: 12,5) is correlated with the reduction of the number of bovine embryo recovered

by donor, although the embryo quality has improved. However, there is no studies about the effect of Folltropin® on the *in vitro* culture of preantral follicles.

Regarding follicular growth, we verified that after seven days, oocyte diameter was significantly higher after culture with 1000 ng/mL of Folltropin® when compared with the other treatments. Oktay *et al.* (1997) reported that the expression of FSH receptors developed progressively during the transition from primordial to primary and secondary follicles. The presence of FSH receptors in granulosa cells suggests that FSH can promote follicular development and growth. On the other hand, in our study, Folltropin® at a concentration of 100 ng/mL decreased follicular diameter from day one to seven of culture. We suggest that this occurs due to oocyte degeneration during the culture period, since in the preantral phase, the oocyte is more sensitive to degeneration than granulosa cells (Silva, *et al.*, 2002).

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 from non-cultured (control) fragments and from those cultured during seven days with Stimufol® were ultrastructurally normal, thus confirming the results obtained in the histological studies. It is important to emphasize that follicles cultured with Stimufol® for seven days had a slight separation between the oocyte and granulosa cells. This finding was also observed in swine preantral follicles cultured *in vitro* (Lucci, personal communication), which did not necessarily indicates follicular degeneration.

In conclusion, FSH preparations affect differently the performance of *in vitro* culture of caprine preantral follicles. Stimufol® was better to preserve follicular viability while Folltropin® was more efficient to promote follicular activation and oocyte growth.

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### References

- Baldassarre H, Karatzas CN (2004). Advanced assisted reproduction technologies (ART) in goats. *Animal Reproduction Science* **82–83**: 255-266.

- Beckers JF (1987). Isolation and use of a porcine FSH to improve the quality of superovulation in cattle. *Theriogenology* **27**: 213.
- Bényei B, Barros CCW (1999). Efeito da superovulação sobre o desempenho de bovinos doadores de embrião importado de clima temperado para clima tropical nos dois primeiros anos de adaptação. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia* **52**: 366-371.
- Bonnet-Garnier A, Lacaze S, Beckers JF, Berland HM, Pinton A, Yerle M, Ducos A (2008). Meiotic segregation analysis in cows carrying the t(1;29) Robertsonian translocation. *Cytogenetic and Genome Research* **120**: 91-96.
- Bousfield GR, Butnev VY, Bidart JM, Dalpathado D, Irungu J, Desaire H (2008). Chromatofocusing Fails To Separate hFSH Isoforms on the Basis of Glycan Structure. *Biochemistry* **47**: 1708-1720.
- Calder MD, Caveney AN, Smith LC, Watson AJ (2003). Responsiveness of bovine cumulus-oocyte-complexes (COC) to porcine and recombinant human FSH, and the effect of COC quality on gonadotropin receptor and Cx43 marker gene mRNAs during maturation *in vitro*. *Reproductive Biology and Endocrinology* **14**: 1-12.
- Cecconi S, Barboni B, Coccia M, Mattioli M (1999). *In vitro* development of sheep preantral follicles. *Biology of Reproduction* **60**: 594-601.
- Closset J, Hennen G (1989). Biopotency of highly purified porcine FSH and human LH on gonadal function. *Journal of Endocrinology* **120**: 89-96.
- Cognie Y (1999). State of the art in sheep-goat embryo transfer. *Theriogenology* **51**: 105-116.
- Eppig JJ (2001). Oocyte control of ovarian follicular development and function in mammals. *Reproduction* **122**: 829-838.
- Fiona TH, Ethier JF, Shimasaki S, Vanderhyden BC (2005). Follicle-Stimulating Hormone Regulates Oocyte Growth by Modulation of Expression of Oocyte and Granulosa Cell Factors. *Endocrinology* **146**: 941-949.
- 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*. *Biology of Reproduction* **62**: 1322-1328.
- Hulshof SCJ, Figueiredo JR, Beckers JF (1994). Isolation and characterization of preantral follicles from foetal bovine ovaries. *Veterinary Quarterly* **16**: 78-80.
- Joyce IM, Pendola FL, Wigglesworth K, Eppig JJ (1999). Oocyte regulation of Kit ligand expression in mouse ovarian follicles. *Development Biology* **214**: 342-53.
- Mao J, Wu G, Smith MF, McCauley TC, Cantley TC, Prather RS, Didion BA, Day BN (2002). Effects of culture medium, serum type, and various concentrations of follicle-stimulating hormone on porcine preantral follicular development and antrum formation *in vitro*. *Biology of Reproduction* **67**: 1197-1203.
- Matos MHT, Lima-Verde IB, Luque MCA, Maia Jr JE, Silva JRV, Celestino JJH, Martins FS, Bão SN, Lucci CM, Figueiredo JR (2007). Essential role of follicle stimulating hormone in the maintenance of caprine preantral follicle viability *in vitro*. *Zygote* **15**: 173-182.
- Mazouz A, Lotfi N, Elaich R, Lakhdi H, Hachi A, Elaide L (1996). La technique de transfert d'embryons bovins chez les éleveurs moyen d'accroître le progrès génétique. *Reproduction et production laitière*: 271-277.
- McGee E, Spears N, Minami S, Hsu SY, Chun SY, Billig H, Hsueh AJW (1997). Preantral ovarian follicles in serum-free culture: suppression of apoptosis after activation of the cyclic guanosine 3'-5'-monophosphate pathway and stimulation of growth and differentiation by follicle-stimulating hormone. *Endocrinology* **138**: 2417-24.
- Nilsson EE, Skinner MK (2001). Cellular interactions that control primordial follicle development and folliculogenesis. *Journal of the Society for Gynecologic Investigation* **8**: 17-20.
- Nuttinck F, Collete L, Massip A (1996). Histologic and autoradiographic study of the *in vitro* effects of FGF-2 and FSH on isolated bovine preantral follicles: preliminary investigations. *Theriogenology* **45**: 1235-1245.
- Oktay K, Briggs D, Gosden RG (1997). Ontogeny of follicle-stimulating hormone receptor gene expression in isolated human ovarian follicles. *Journal of Clinical Endocrinology & Metabolism* **82**: 3748-3751.
- O'Shaughnessy PJ, Dudley K, Rajapaksha WR (1996). Expression of follicle stimulating hormone-receptor mRNA during gonadal development. *Molecular and Cellular Endocrinology* **125**: 169-175.
- Salvetti P, Theau-Clément M, Beckers JF, Hurtaud J, Guérin P, Neto V, Falières J, Joly T (2007). Effect of the luteinizing hormone on embryo production in superovulated rabbit does. *Theriogenology* **67**: 1185-1193.
- Saraiva MVA, Celestino JJH, Chaves RN, Martins FS, Bruno JB, Lima-Verde IB, Matos MHT, Silva GM, Porfirio EP, Bão SN, Campello CC, Silva JRV, Figueiredo JR (2008). Influence of different concentrations of LH and FSH on caprine primordial ovarian follicle development *in vitro*. *Small Ruminant Research* **78**: 87-95.
- Silva JRV, Ferreira MAL, Costa SHF, Santos RR, Carvalho FCA, Rodrigues APR, Lucci CM, Bão SN, Figueiredo JR (2002). Degeneration rate of preantral follicles in the ovaries of goats. *Small Ruminant Research* **43**: 203-209.
- Tecnopec (2008). <http://www.tecnopec.com.br/index.php?aid=51>. Accessed March 5, 2008.
- Tsai-turton M, Luderer U (2006). Opposing effects of glutathione depletion and follicle-stimulating hormone on reactive oxygen species and apoptosis in cultured preovulatory rat follicles. *Endocrinology* **147**: 1224-1236.
- Ulloa-Aguirre A, Timossi C, Barrios-de-Tomasi J, Maldonado A, Nayudu P (2003). Impact of carbohydrate heterogeneity in function of FSH: studies derived from *in vitro* and *in vivo* models. *Biology of Reproduction* **69**: 379-389.
- Wright CS, Hovatta O, Margara R, Trew G, Winston RML, Franks S, Hardy K (1999). Effects of follicle-stimulating hormone and serum substitution on the *in-vitro* growth of human ovarian follicles. *Human Reproduction* **14**: 1555-1562.

