



Caprine ovarian follicle requirements differ between preantral and early antral stages after IVC in medium supplemented with GH and VEGF alone or in combination



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ABSTRACT

The aim of the present study was to evaluate the effect of growth hormone (GH) and vascular endothelial growth factor (VEGF) added alone, sequentially or in combination, in the presence of insulin at physiological concentration (10 ng/mL) on the IVC of two different follicular categories: preantral (experiment 1; Exp.1) and early antral (experiment 2; Exp.2). Isolated follicles were individually cultured for 24 (Exp.1) and 18 days (Exp.2) in the following treatments: α MEM⁺ (Control), or Control medium supplemented with 50 ng/mL GH (GH), 100 ng/mL VEGF (VEGF), the combination of both (GH + VEGF), GH during the first 12 days and VEGF from Day 12 until the end of the culture (GH/VEGF) and vice versa (VEGF/GH). At the end of the culture, cumulus-oocyte complexes from *in vitro*-grown follicles were recovered and subjected to IVM. The following end points were evaluated: Follicle morphology, growth rates and antrum formation, production of estradiol, progesterone and testosterone, oocyte viability and meiotic stage, as well as relative expression of *LHR*, *Amh*, *HAS2*, *PTGS2*, *CYP17*, *CYP19A1*, and *3 β HSD*. A considerable amount of viable fully grown oocytes were recovered after the IVC of early antral follicles in all treatments. Nevertheless, the GH treatment presented the highest percentage of fully grown oocytes (60%), mean oocyte diameter ($117.74 \pm 2.61 \mu\text{m}$), and meiotic resumption (50%). Furthermore, GH treatment produced higher ($P < 0.05$) rates of metaphase II oocytes than all the other treatments, and similar *LHR*, *Amh*, and *PTGS2* transcript levels to *in vivo*. Contrary to early antral follicles, preantral follicles were not affected by medium supplementation. In conclusion, the addition of GH to a culture medium containing physiological concentrations of insulin improves oocyte growth and maturation after the IVC of goat early antral follicles.

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1. Introduction

It is known that mammalian ovaries contain a large population of oocytes, being the vast majority enclosed in preantral follicles (PFs; i.e., primordial, primary, and secondary follicles). However, 99.9% of them never reach

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ovulation, but rather, become atretic during their growth or maturation. A developing biotechnology called *in vitro* follicle culture (IVFC) might increase the number of potentially fertilizable oocytes by recovering, preserving, and *in vitro* culturing those follicles, for the purpose of prevention of atresia [11].

The IVFC biotechnology has succeeded in mice, where birth of live offspring was obtained after fertilization of oocytes from *in vitro*–cultured PFs [2]. On the other hand, in large animals, such an achievement has not been reported. Instead, the results have been limited to the production of a low and variable number of metaphase II (MII) oocytes and embryos [3–6].

In caprine, the highest MII oocyte rate obtained from PFs grown *in vitro* so far is 29.4% [7], and the latest stage of embryo development attained is morulae, which was also the only embryo produced in that study [5]. Those results were obtained using culture medium supplemented with vascular endothelial growth factor (VEGF) and growth hormone (GH), respectively. Both culture media contained high insulin concentration (10 $\mu\text{g/mL}$) and FSH in increasing concentrations (from 100 to 1000 ng/mL). Nonetheless, the proper balance between FSH and insulin concentration *in vitro* is still under discussion. Chaves et al. [8] reported that a lower concentration of insulin (10 ng/mL) was more efficient in promoting meiotic resumption. This finding is consistent with the idea that the combination of both, FSH and insulin in high concentration, impacts oocyte competence and induces abnormal patterns of expression of genes related to oocyte maturation and cumulus cells differentiation, as already suggested by other authors [9,10].

Despite the improvements made on IVFC in caprine in the last years, the current *in vitro* systems are still unable to produce an amount of MII oocytes similar to those from follicles grown *in vivo*. One possible reason could be that follicle requirements are continuously changing throughout the culture period. Magalhães-Padilha et al. [11] pointed out this by microarray analysis; they observed the temporal changes in transcriptional profiles of secondary and early antral follicles in caprine ovaries. In this study, gene expression profiles showed that three major metabolic pathways (lipid metabolism, cell death, and hematological system) were significantly differentiated between the two follicle stages. Consequently, we infer that secondary and early antral follicles behave differently under the same culture conditions; hence, they have different supplementation needs *in vitro*. This fact could be a key factor to develop a future culture system that reproduces complete folliculogenesis *in vitro*.

Based on the previous information, the aim of this study was to test for the first time the effects of GH and VEGF alone, in association or sequentially, in the presence of low concentration of insulin (10 ng/mL) and absence of FSH on two different follicular categories: caprine preantral and early antral follicles. The following end points will be evaluated: (1) follicular growth and morphology; (2) oocyte maturation and viability; (3) estradiol (E2), progesterone (P4), and testosterone (T) production; and (4) gene expression levels of *CYP17*, *CYP19A1*, and *3 β HSD* in follicle walls and *HAS2*, *PTGS2*, *Amh*, and *LHR* in cumulus cells.

2. Materials and methods

All experiments were performed according to the recommendations of the Committee of Animal Handling and Ethical Regulation from the State University of Ceara, Fortaleza, Ceara, Brazil.

2.1. Chemicals and media

Unless mentioned otherwise, the reagents and chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, Mo, USA).

2.2. Source of ovaries

Ovaries from 48 adult mixed-breed goats (1- to 3-year old) were collected at a local abattoir and used to perform experiments 1 (24 pairs) and 2 (24 pairs). Immediately after the slaughter, the ovaries were immersed in 70% alcohol, followed by two washes in minimum essential medium (MEM) plus HEPES (MEM-HEPES), supplemented with 100- $\mu\text{g/mL}$ penicillin and 100- $\mu\text{g/mL}$ streptomycin, and then transported to the laboratory at 4 °C [12] within 3 hours since they were collected.

2.3. Isolation and selection of caprine preantral and early antral follicles

In the laboratory, the surrounding fat and connective tissue were removed from the ovaries. Ovarian cortical slices (1-mm thick) were cut using a surgical blade and placed in holding medium (MEM-HEPES), and then, preantral and early antral follicles were isolated. Two experiments were performed: experiment 1 (Exp.1) with PFs and experiment 2 (Exp.2) with early antral follicles. For Exp.1, PFs (100–200 μm) were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan), manually dissected from the slices of ovarian cortex using 26-gauge (26-G) needles, and transferred to the culture medium for further evaluation of follicular quality. Follicles with a visible central oocyte, surrounded by two or more granulosa cell layers and with an intact basement membrane and no antral cavity, were selected for IVC. In Exp.2, the procedure was the same as in the previous experiment, with the difference that only early antral follicles (300–400 μm) were selected for IVC.

2.4. *In vitro* culture of caprine preantral and early antral follicles

Immediately after follicle isolation, the isolated follicles from all animals were pooled and randomly assigned to the six treatments, so every treatment is supposed to have follicles from all animals (Fig. 1). A total amount of 240 preantral (Exp.1) and 253 early antral follicles (Exp.2) were used in experiments 1 and 2, respectively. Regardless of the experiment, follicles were individually cultured in 100 μL drops of culture medium on Petri dishes under mineral oil (60 $\times$ 15 mm; Corning, USA) for 24 days (Exp.1) or 18 days (Exp.2). Since in our culture system, antrum formation begins by Day 6 of culture, for Exp.2, whose follicles already had antrum at Day 0, we decided to diminish the culture

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