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Effect of morphological integrity, period, and type of culture system on the *in vitro* development of isolated caprine preantral follicles

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ABSTRACT

The aims of this study were the following: (1) to define an optimal period for the IVC of isolated caprine preantral follicles, (2) to verify the relationship between follicular morphology (intact, extruded, and degenerate follicles) and estradiol production, and (3) to evaluate the effects of the bidimensional (2D) and three-dimensional (3D) culture systems on the *in vitro* development of caprine preantral follicles. Three experiments were performed. In experiments 1 and 2, the isolated secondary follicles were cultured for 18, 24, and 30 days or 30, 36, and 42 days, respectively. In experiment 3, the optimal culture period from experiment 2 was used for 2D and 3D culture systems. After culture, the oocytes were submitted to IVM. The morphological integrity, antral cavity formation rates, follicular diameter, presence of healthy, grown oocytes ($\geq$110 μm), rates of resumption of meiosis, and estradiol concentrations were evaluated. In experiment 1, the percentage of oocytes that resumed meiosis was higher in oocytes cultured for 30 days (48.84%) than in oocytes cultured for 18 and 24 days (15% and 20.93%, respectively). In experiment 2, the percentage of oocytes that resumed meiosis was significantly higher in oocytes cultured for 30 and 36 days (47.5% and 50%, respectively) than in oocytes cultured for 42 days (20%). The estradiol concentrations on Day 12 of culture were similar for normal and extruded follicles and higher than those observed in degenerate follicles at the end of the culture period. In conclusion, the 36-day culture period resulted in the highest rates of meiosis resumption. In addition, because the loss of follicular integrity affects the patterns of estradiol production, follicular integrity is a good predictor of follicular quality.

1. Introduction

The IVC of preantral follicles represents a promising reproductive biotechnology that can be used for the IVM of a large number of immature oocytes. Oocytes from IVC can be used for the *in vitro* production of embryos from high-value or endangered animals. Currently, encouraging results have been obtained with the IVC of preantral follicles for embryo production in farm animals [1–3]. However, low rates of oocyte maturation have limited these results to a low and variable number of embryos.

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In an attempt to develop an efficient medium for total follicular development, several authors have tested the effects of hormones [4,5] and growth factors [6] on the IVC of isolated goat preantral follicles. In most of these studies, the culture period was 18 days. However, it is unclear whether an 18-day culture period is ideal for total caprine follicular development. Gupta et al. [7] described the production of buffalo embryos after approximately 100 days of culture of secondary follicles. Thus, studies testing longer-term cultures of caprine preantral follicles are necessary to increase the rates of oocyte maturation and subsequent embryo production.

An important factor for follicular culture is the system in which the follicles (whether follicles included in the ovarian cortex (*in situ*) [8] or isolated follicles [9]) develop. The IVC of isolated follicles can be performed in a two- (2D) or three-dimensional (3D) culture system. In the 3D culture system, the follicle is inside of a substrate that maintains the follicular architecture. Magalhães et al. [3] described the production of caprine embryos following the IVC of preantral follicles in the presence of GH in the 2D culture system. The 3D culture system has supported the development of oocytes and follicles in mice and non-human primates [10,11]. However, no studies have compared the 2D and 3D culture systems for the IVC of preantral goat follicles.

Another point of controversy in the literature concerns the necessity of maintaining follicular integrity to ensure follicular functionality. Enzymatically isolated follicles can lose their basement membrane [12], and mechanically isolated follicles may even extrude their oocytes during culture. However, it is not known whether the production of steroids such as estradiol [13] can predict follicular fate during culture or whether this production is related to the maintenance of follicular integrity.

Therefore, the aims of this study were to (1) define an optimal period for the IVC of isolated caprine preantral follicles that allows good rates of follicular viability, antrum formation, and resumption of oocyte meiosis; (2) investigate the relationship between follicular morphology (intact, extruded, and degenerated follicles) and estradiol concentrations; and (3) evaluate the effects of 2D and 3D culture systems on the *in vitro* development of caprine preantral follicles.

2. Materials and methods

2.1. Collection of ovaries

Twenty pairs of ovaries from mixed-breed goats were collected from a local abattoir and used for each experiment. After collection, the ovaries were washed in 70% ethanol and rinsed twice in Minimum Essential Medium-HEPES supplemented with penicillin (100 μg/mL) and streptomycin (100 μg/mL). The ovaries were transported to the laboratory in Minimum Essential Medium-HEPES at 4 °C within approximately 1 hour [14].

2.2. Experimental protocol

Three experiments were conducted. Experiment 1 aimed to analyze the effects of different durations of IVC (18, 24, and 30 days) on the morphological integrity and antrum formation rate of follicles, follicular and oocyte diameters, and the resumption of meiosis in preantral follicles cultured *in vitro*. Because a 30-day culture period was found to be optimal in experiment 1, that is, produced better results in terms of the resumption of oocyte meiosis, a 30-day culture period was used as a control in experiment 2 and compared with longer culture periods (36 and 42 days). In other words, experiment 2 aimed to verify the effects of 30, 36, and 42 days of IVC on all end points analyzed in experiment 1. Experiment 3 aimed to compare the effects of 2D and 3D culture systems on the IVC of preantral follicles using the optimal culture period obtained in experiment 2 (36 days). Irrespective of the experiment, 40 isolated secondary follicles were cultured for each treatment.

2.3. Follicular isolation

In the laboratory, the fat and connective tissue surrounding the ovary was removed. Fragments of ovarian cortex (1–2 mm thick) were cut from the ovarian surface with a surgical blade under sterile conditions. Subsequently, the fragments of ovarian cortex were placed in fragmentation medium consisting of Minimum Essential Medium-HEPES. Secondary follicles between 150 and 250 μm in diameter were identified under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and manually isolated using 26-G needles coupled to 1 mL syringes. After isolation, only follicles with a normal morphology were selected, that is, those with a centrally located spherical oocyte surrounded by two or more compact layers of granulosa cells with no apparent damage to the basal membrane or antral cavity.

2.4. In vitro culture of goat preantral follicles

After selection, the follicles were cultured individually in 100-μL droplets of medium under mineral oil in petri dishes (60 × 15 mm diameter; 2D culture system). The culture medium consisted of α-MEM supplemented with 3 mg/mL BSA, 2 mM glutamine, 2 mM hypoxanthine, 10 ng/mL insulin, 5.5 mg/mL transferrin, 5 ng/mL selenium, 50 μg/mL ascorbic acid, 50 ng/mL of GH, and sequential FSH (Day 0–6: 100 ng/mL; Day 6–12: 500 ng/mL; Day 12–42: 1000 ng/mL). The cells were cultured for 18, 24, and 30 days (experiment 1), for 30, 36, and 42 days (experiment 2) or for 36 days (experiment 3) in an incubator at 39 °C and 5% CO_2.

2.5. Two-dimensional and three-dimensional culture systems

Because the highest oocyte recovery rates (oocytes ≥ 110 μm) were obtained with the 36-day culture period, this culture period was used in the 2D and 3D culture systems. The goat ovaries were collected and transported to the laboratory as previously described. For the 3D culture system, isolated follicles were transferred to 7-μL drops of a 0.25% solution of sodium alginate (FMC Bio Polymers, Philadelphia, PA, USA). Subsequently, the beads were polymerized in a solution of 50 mM $CaCl_2$ and 140 mM NaCl for 1 minute. Each follicle encapsulated in alginate was transferred to an individual well of a 48-well plate