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Impact of insulin concentration and mode of FSH addition on the *in vitro* survival and development of isolated bovine preantral follicles

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The insulin and FSH are two important substances in the folliculogenesis process. Thus, the hypothesis of this experiment is that insulin concentration and the form of FSH addition affect the *in vitro* survival, growth, and estradiol production after culture of isolated bovine preantral follicles. The effects of insulin concentration (experiment 1) and the influence of both fixed and sequential concentrations of FSH (experiment 2) on the *in vitro* survival and development of bovine preantral follicles were investigated in this study by IVC for 18 days. In experiment 1, on Day 18 of culture, the addition of insulin at all concentrations promoted follicular survival rates significantly higher than that of the control, with the 10-ng/mL insulin treatment showing values significantly higher than the other treatments. The addition of 5- and 10-ng/mL insulin promoted higher follicular growth than the control and other treatments. In experiment 2, FSH 100 had a higher percentage of follicular viability compared with the control. FSH 100 produced follicle diameters significantly higher than those of the control and FSH seq. treatment. Estradiol levels in the presence of FSH (fixed concentration) were significantly higher than the other treatments. In conclusion, the association of insulin (10 ng/mL) and fixed concentration FSH (100 ng/mL) provides high rates of survival, growth, and estradiol production in bovine preantral follicles.

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1. Introduction

The *in vitro* follicle culture using either 2D [1] or 3D [2] systems provides a valuable *in vitro* model for studying the regulation of the folliculogenesis process, which involves complex interactions among the growth factors and hormones. The process of early folliculogenesis is mainly regulated by the intraovarian factors; however, gonadotropins also play a role in modulating this mechanism [3]. In recent years, the role of FSH in preantral follicles has been widely studied, characterizing the effects of this gonadotropin on *in vitro* follicular survival and growth [4,5]. Moreover, studies have suggested that other hormones related to cell metabolism, such as insulin, are also essential during ovarian folliculogenesis in mammals [6].

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Insulin is a common constituent of IVC medium for isolated preantral follicles in many species including bovine [7]. However, different insulin concentrations (10 ng/mL [8,9]; 20 ng/mL [10]; and 10 μg/mL [7,11]) have been used for IVC of bovine preantral follicles. Despite the importance of insulin for follicle survival and development [7–10], to the best of our knowledge, there are no studies comparing the efficiency of different insulin concentrations on IVC of bovine preantral follicles under the same experimental condition. It is important to emphasize that relevant results including the *in vitro* embryo and offspring production from *in vitro* preantral follicle culture have been reported using either low- (10 ng/mL: ovine) [12] and high- (10 μg/mL: caprine) [2] insulin concentrations. Like insulin, FSH has been extensive used as medium supplement during IVC of preantral follicles either in fixed [13] or increasing concentrations [14]. It is believed that FSH requirement increases throughout folliculogenesis [14], and *in vitro* study performed in goats reported that the percentage of follicle survival and development was improved in the presence of increasing FSH concentration than in the presence of fixed concentration. However, most authors use a fixed FSH concentration during IVC of preantral follicles. Our hypothesis is that insulin concentration and the form of FSH addition affect the *in vitro* survival, growth, and estradiol (E2) production after culture of isolated bovine preantral follicles.

Thus, the aim of the present study was to determine the effect of insulin concentrations on the IVC of isolated bovine preantral follicles and to evaluate the influence of the form that the rFSH is added (fixed and increasing FSH concentrations) on the survival, growth, hormone production, and transmission electron microscopy (TEM) of isolated bovine preantral follicles cultured in a three-dimensional system.

2. Materials and methods

2.1. Source of ovaries

Ovaries (n = 44) were collected from adult cross-bred cows from a local slaughterhouse to perform the experiments. Some of the ovaries (n = 26) were used in experiment 1, and the remaining (n = 18) were used in experiment 2. Immediately after slaughter, the ovaries were removed and washed with 70% alcohol for 10 seconds, followed by two washes in Minimum Essential Medium (MEM; Sigma Chemical Co., St. Louis, MO, USA) buffered with HEPES (MEM–HEPES) and supplemented with penicillin (100 μg/mL) and streptomycin (100 μg/mL). The ovaries were transported to the laboratory at 4 °C within 1 hour.

2.2. Preantral follicle isolation and selection

For follicular isolation, the ovaries were subjected to a microdissection procedure, as previously described [15]. Briefly, fine fragments were obtained from the ovarian cortex (1–2 mm thick) using a sterile scalpel blade. The ovarian cortex slices were placed in a fragmentation medium consisting of HEPES-buffered MEM. Bovine preantral follicles exceeding 150 μm in diameter were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and manually dissected from strips of the ovarian cortex using 26-gauge (26 G) needles. After isolation, follicles were transferred to 100-μL drops containing fresh medium under mineral oil to further evaluate follicular quality. Follicles with a visible oocyte surrounded by granulosa cells with an intact basement membrane and without an antral cavity were selected for IVC.

2.3. Morphological evaluation of follicular development

On Days 0, 6, 12, and 18 of culture, the follicles were classified according to their morphological aspect, and only those showing an intact basement membrane, bright and homogeneous granulosa cells, and the absence of morphological signs of degeneration, such as darkness of the oocytes and surrounding cumulus cells, or misshapen oocytes, were classified as viable follicles. The follicles were also evaluated and classified according to the presence or the absence of an antral cavity, which was defined as a visible translucent cavity within the granulosa cell layers. Moreover, follicular diameters were measured, only in healthy follicles, using the mean of two perpendicular measurements of each follicle with an ocular micrometer (100 × magnification) inserted into a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan).

2.4. Experiment 1: Two-dimensional in vitro culture of bovine preantral follicles using different concentrations of insulin

The selected follicles were individually cultured in 96-well plates (Corning Life Sciences, Corning, NY, USA) containing 150 μL of medium per well for 18 days at 38.5 °C with 5% CO_2 in air. The base medium consisted of TCM199 supplemented with HEPES, 1% bovine serum albumin (MP Biomedicals, Solon, OH, USA), 3-mM glutamine (Sigma Chemical Co., St. Louis, MO, USA), 2.5-μg/mL transferrin (Sigma Chemical Co., St. Louis, MO, USA), 4-ng/mL selenium (MP Biomedicals, Solon, OH, USA), 50-μg/mL ascorbic acid (Sigma Chemical Co., St. Louis, MO, USA), 100-ng/mL activin-A (Cell Sciences, Canton, MA, USA), and antibiotics (0.1-mg/L penicillin and 0.1-mg/L streptomycin; Sigma Chemical Co., St. Louis, MO, USA). The base medium was used in the absence (control) or presence of insulin (Sigma Chemical Co., St. Louis, MO, USA) at concentrations of 5 ng/mL, 10 ng/mL, 5 μg/mL, and 10 μg/mL.

2.4.1. Assessment of follicular viability by confocal microscopy

Fluorescence microscopy was used to analyze the viability of oocytes isolated from caprine preantral follicles after 18 days of culture. At the end of the culture period (Day 18), the follicles were incubated in 100-μL D-PBS containing 4-μmol/L calcein-AM and 2-μmol/L ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany) for 15 minutes at 37 °C [2]. After exposure to the fluorescent markers, the follicles were washed in D-PBS and mounted on slides with coverslips for observation by laser confocal microscopy using an LSM 710 microscope (Zeiss, Oberkochen, Germany). The emitted fluorescent signals of calcein-AM and ethidium homodimer-1 were

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