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Effect of culture medium replacement protocol on the in vitro development of isolated caprine secondary follicles

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ABSTRACT

The aim of this study was to verify the influence of three different protocols for medium refreshing on the in vitro culture of isolated caprine preantral follicles. Independently of the protocol, preantral follicles were individually cultured for 18 days, the initial volume of medium was 25 μl, and the interval of medium refreshing was every two days. The protocols tested were: T1 (Control) – refreshing of 15 μl (removal of 15 μl of culture medium followed by the addition of the same volume of fresh medium), maintaining a final volume of 25 μl, T2 – only the addition of 5 μl of fresh medium every two days (the medium volume increases 5 μl for each change up to a final volume of 65 μl at day 18), and T3 – initial removal of 15 μl of medium in the first change, with addition of 20 μl of fresh medium (net increase of 5 μl in the final volume at each change). In the subsequent changes for T3, the amount of medium added in the previous change was removed, followed by the addition of the same volume plus 5 μl fresh medium (as occurred for T2 the final volume at day 18 is also 65 μl). Analyses of survival, diameter and antrum formation, as well as the rate of daily follicular growth were performed every 6 days. At the end of the culture period, normal oocytes ≥110 μm were destined for in vitro maturation (IVM). The results showed that only T2 (addition without removal of medium) maintained follicular survival until the end of the culture period. In day 18, both follicular diameter and the rate of daily growth was similar in T2 and T3 (Removal + Addition of medium), which were both higher than in T1 (Partial change). Moreover, T2 obtained a greater percentage of oocytes >110 μm destined for IVM and was the only treatment that achieved an oocyte in the telophase-I stage. In conclusion, periodic addition of medium is recommended because it is more practical, maintains survival and promotes the development of caprine preantral follicles in vitro.

1. Introduction

The ovaries of mammals contain a large number of oocytes enclosed in preantral follicles. In vivo, follicles develop through primordial, primary and secondary stages (preantral follicle phase) before acquiring an antral cavity. In primordial and primary follicles, the granulosa cells in beginning of proliferation are more resistant to degenera-

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tion than oocytes (Braw-tal and Yossefi, 1997). However, in secondary follicles, both oocyte and granulosa cells are equally affected. The increase in oocyte sensitivity in advanced follicles may be due to the significant morphological alterations in this compartment or the need for greater nutritional support (Silva et al., 2002). The in vitro culture of secondary follicles up to antral stage is very important to provide information about follicular requirements during the late preantral follicle phase as well as during the antral phase. Due to these differences among follicular categories, the development of culture systems that support complete follicular growth in vitro is necessary. Such culture systems depend on the physical and biochemical conditions used, which may limit the diffusion of nutritional gradients, thus providing an adequate environment for the growth and development of competent oocytes (Hastshorne, 1997).

The development of protocols for the periodic replacement of culture medium (change of medium) is extremely important for in vitro culture because cultured cells produce substances that are favorable and/or harmful to their survival. For instance, the amino acids present in culture medium can be metabolized at 37 °C and thus, stimulate the production of ammonia, which may be deleterious for the cells in culture. To avoid the accumulation of this compound and other toxic products, some authors have suggested that for the culture of embryos, medium replacement should occur at least every 72 h (Gardner et al., 1994; Trounson et al., 1994).

For advanced preantral follicles, partial replacement of the medium is most commonly used for growth in culture. This procedure provided satisfactory results, such as the production of embryos in swine (Wu et al., 2001), mouse (Demeestere et al., 2002) and buffalo (Gupta et al., 2008). In caprine, the partial change of medium allowed the formation of an antral cavity in follicles cultured in a group (Huanmin and Yong, 2000) and meiosis resumption, reaching metaphase I in isolated follicles cultured in an atmosphere of 20% oxygen (Silva et al., 2010). Other studies cultured mouse preantral follicles in 10 μl drops wherein the replacement of the medium was performed by the addition of 10 μl in day 1 of culture and subsequent replacement of half of the medium (partial change). This protocol promoted follicular growth and production of matured oocytes in vitro (Mousset-Siméon et al., 2005; Lee et al., 2007).

Despite the encouraging results obtained with the culture of isolated follicles, the establishment of an efficient protocol of medium replacement is still necessary to produce a greater number of caprine oocytes competent to resume meiosis in vitro. Thus, the aim of the present study was to observe the influence of different protocols of partial replacement and/or periodic addition of medium in the in vitro culture of advanced isolated preantral follicles from caprine ovaries.

2. Materials and methods

2.1. Source of chemicals and ovaries

Unless mentioned otherwise, the culture media and other chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, USA). Ovaries ($n = 24$) were collected at a local slaughterhouse from 12 adult (ages 1–3 years) mixed-breed goats. Immediately postmortem, the ovaries were washed in 70% alcohol for approximately 10 s, followed by two rinses in Minimum Essential Medium with HEPES (MEM HEPES) supplemented with 100 μg/ml penicillin and 100 μg/ml streptomycin. The ovaries were transported within 1 h to the laboratory in 15 ml tubes containing washing medium at 4 °C (Chaves et al., 2008).

2.2. Isolation and selection of caprine preantral follicles

In the laboratory, the fat tissue and ligaments surrounding the ovaries were stripped off, and caprine ovarian cortical slices (1 mm thick) were cut from the ovarian surface using a surgical blade under sterile conditions. Then the ovarian fragment was placed in a fragmentation medium consisting of MEM HEPES. Caprine preantral follicles that were approximately ≥150 μm in diameter were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and manually dissected from strips of the ovarian cortex using 27.5 gauge (27.5 G) needles. After isolation, follicles were transferred to 100 μl drops containing fresh medium to further evaluate the follicular quality. Follicles with a visible oocyte that were surrounded by two or more layers of granulosa cells, with an intact basement membrane and no antral cavity were selected for culture (Fig. 1A).

2.3. Culture of caprine preantral follicles

After selection, follicles were individually cultured in 25 μl drops (initial volume for all protocols tested) of culture medium under mineral oil in Petri dishes (60 mm × 15 mm, Corning, USA). The basic culture medium consisted of α-MEM (pH 7.2–7.4) supplemented with 1.25 mg/ml bovine serum albumin (BSA), ITS (insulin 10 μg/ml, transferrin 5.5 μg/ml and selenium 5 ng/ml), 2 mM glutamine, 2 mM hypoxantine, 50 μg/ml ascorbic acid, and 1000 ng/ml recombinant Follicle Stimulating Hormone (rFSH®, Nanocore, Brazil). Preantral follicles were individually distributed in microdrops according to the protocol of medium replacement used (treatments): T1 (Control) – Partial change of 15 μl (removal of 15 μl of culture medium followed by the addition of the same volume of fresh medium, maintaining a final volume of 25 μl, T2 – only addition of 5 μl of fresh medium at each change (final volume of 65 μl), and T3 – initial removal of 15 μl of medium in the first change, with addition of 20 μl of fresh medium (increase in 5 μl in the final volume at each change). In the subsequent changes for T3, the amount of medium added in the previous change was removed, followed by the addition of the same volume plus 5 μl fresh medium (final volume of 65 μl). Independent of the treatment, addition and/or removal of the culture medium occurred every two days with the medium being incubated for 1 h prior to use. Incubation was carried out at 39 °C, in 5% CO_2 in air for 18 days after which follicles were recovered for in vitro maturation. The experiment was replicated four times, and at least 30 follicles were used for each treatment.

2.4. Morphological evaluation of follicle morphology and development

During and after culture, follicles were classified according to their morphological characteristics, and those showing morphological signs of degeneration, such as darkness of the oocytes and the surrounding granulosa cells, misshapen oocytes, rupture of the basement membrane and/or oocyte extrusion were classified as degenerated. Analyses of follicular viability as well as the medium replacement were performed every two days of culture. The rate of daily follicular growth was calculated by the variation of follicular diameter (diameter of viable follicles after 18 days of culture minus diameter on day 0) divided by the period of culture. Follicular diameter was measured only in healthy follicles from the basement membrane, in the x and y dimensions (90°), using an ocular micrometer (100× magnification) inserted into a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) every six days of culture (at days 0, 6, 12 and 18 of culture). Antral cavity formation was defined as a visible translucent cavity within the granulosa cell layers (Fig. 1B).

2.5. Oocyte recovery rate (≥110 μm in diameter) from in vitro grown caprine preantral follicles

After 18 days of culture, all of the healthy follicles were carefully and mechanically opened with 27.5 G needles under a stereomicroscope for oocyte recovery. Only oocytes ≥110 μm, with a homogeneous cytoplasm that were surrounded by at least one compact layer of cumulus cells were selected for in vitro maturation (IVM). The recovery rate was