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Ultrastructural and cytochemical characterization of follicular cell types in bovine (*Bos taurus*) cumulus–oocyte complexes aspirated from small and medium antral follicles during the estrus cycle

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ABSTRACT

Stereological quantitative methods have revealed the presence of three distinct follicular cell populations (C1–C3) in bovine cumulus–oocyte complexes. Type C3 cells became largely predominant from metestrous to proestrous, with a simultaneously large decrease in the other two cell types. To further characterize these cumulus cell types, cumulus–oocyte complexes from small (1–4 mm) and medium (4–8 mm) antral follicles (category 1: with a compact multilayered cumulus and a homogeneous ooplasm) were aspirated from ovaries of Holstein–Friesian cows and processed for electron microscopy, ultrastructural cytochemical detection of glycogen and glycoproteins, and immunogold localization of chondroitin sulfate. Each follicular cell type displayed the same ultrastructural characteristics independently of the size of the follicle and the stage of the estrous cycle. Type C1 cells showed morphological characteristics of undifferentiated cells and progressively transformed into type C2 cells. Type C2 cells were characterized by cell extensions, polarized nuclei with evident nucleolar fibrilar centers, steroidogenic characteristics (numerous large lipid droplets, large endoplasmic reticulum vesicles and vacuoles), and synthesis of glycoproteins and chondroitin sulfate by the Golgi apparatus and endoplasmic reticulum. Type C3 cells presented morphologic features of fully differentiated and luteinized cumulus cells. They were characterized by an increase in cytoskeleton filaments, loss of cell extensions and of intercellular junctions, depletion of lipid and glycogen stores, and initiation of glycoprotein and chondroitin sulfate exocytosis. In conclusion, the present study suggests that bovine cumulus cells from small and medium antral follicles follow a complete dynamic functional differentiation process, in which the three cell types seem to correspond to a functional continuum. We identified undifferentiated cells, cells strongly engaged in glycoprotein and proteoglycan synthesis, and cells fully differentiated and secreting.

1. Introduction

In the bovine, 2–3 waves of follicular development take place during the estrous cycle. In each wave, a group of 3–6 follicles is recruited and grow up to 4 mm independently of hormone levels (Gong et al., 1996), becoming responsive to FSH and LH after that period by expressing specific receptors for gonadotrophins (Hunter et al., 2004; Webb et al., 2003). After wave emergence, declining of FSH concentrations induce the selection of one of the follicles for continued growth. The dominant follicle then secretes inhibin and suppresses the growth of the other follicles (subordinate follicles), which thus enter atresia. The

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dominant follicle of the first wave may also undergo atresia (anovulatory wave), and only the dominant follicle of the second wave (in two wave cycles) or of the third wave (in three wave cycles) then proceeds to ovulation (Irving-Rodgers et al., 2002; Singh and Adams, 2000; Van den Hurk et al., 1998).

Bovine oocytes used for in vitro fertilization and embryo transfer are retrieved from immature antral follicles and in vitro matured with FSH and LH (Hasler et al., 1995; Ireland et al., 1979; Leibfried and First, 1979; Shamsuddin et al., 1996; Sirard and Blondin, 1996; Takagi et al., 1998). For this, immature cumulus–oocyte complexes (COCs) with a compact multilayered cumulus and a homogeneous ooplasm (category 1) are chosen as they have been shown to present higher developmental ability (Blondin and Sirard, 1995; Carolan et al., 1996; De Loos et al., 1992; Hazeleger et al., 1995; Hyttel et al., 1986b; Madison et al., 1992).

We have previously applied design-based stereological methods to quantitatively characterize bovine small (1–4 mm) and medium (4–8 mm) antral COCs of category 1 (Calado et al., 2001, 2002, 2003, 2005). Data showed that the volumes of COCs, oocytes and nuclei of oocytes from small antral follicles increased from metestrous to proestrous, whereas those from medium antral follicles displayed no changes. Because a linear correlation between the volumes of the oocyte and nucleus was found only in COCs from medium antral follicles, data suggested that up to a certain follicle size the increase in the volume of the oocyte is non-synchronized with the ongoing expansion of the germinal vesicle and that after some threshold is achieved, both the oocyte and nucleus expand in harmony (stable growth phase). This threshold could actually divide the follicles into largest subordinate and second largest subordinate follicles, as described by Singh and Adams (2000). Results also demonstrated that COCs from distinct estrous stages are structurally different, a finding that might offer an explanation for the highly variable developmental rates seen in vitro (De Loos et al., 1989, 1991; Fair et al., 1995; Hawk and Wall, 1994; Otoi et al., 1997; Younis et al., 1989). Finally, it could also be demonstrated that the cumulus mass contains three different cell populations (C1–C3). The numerical frequency of follicular type C3 cells was shown to increase from metestrous to proestrous, thus becoming the predominant cell type, with data suggesting that follicular type C3 cells derive from type C2 cells and the latter from type C1 cells (Calado et al., 2001, 2002, 2003, 2005).

To further characterize these different functional follicular cell populations, we studied the ultrastructural characteristics of the three follicular cell types of category 1 bovine COCs collected from small and medium antral follicles during metestrous, diestrous and proestrous.

2. Material and methods

2.1. Collection of cumulus–oocyte complexes

Ovaries were collected from Holstein–Friesian cyclic cows at a local slaughterhouse (Calado et al., 2001, 2002, 2003, 2005). The stage of the cycle was selected based on corpus hemorrhagicum/corpus luteum (CL) appearance. Briefly, metestrous ovaries had a CL with a red surface and a recently ruptured follicle (0.5–1.5 cm in diameter); in diestrous ovaries the point of rupture of follicle was covered over and the apex of the bisected CL was red/brown (1.6–2 cm) with a visible surface vasculature, or the bisected CL was orange/yellow (2 cm) with a prominent surface vasculature; and proestrous ovaries showed absent vasculature on CL surface and presence of at least one large ($\geq$10–11 mm in diameter) follicle (Ireland et al., 1979; Singh and Adams, 2000). We randomly selected thirty COCs from the three phases of the estrous cycle and from small and medium antral follicles (1–4 and 4–8 mm of diameter, respectively), in a total of five COCs per group. We avoided the used of more than one COC per animal.

Ovaries were transported to the laboratory within 1.5–3 h at ambient temperature in sterile PBS with 1% (v/v) penicillin/streptomycin (Sigma, Barcelona, Spain) according to other authors (Kacinskis et al., 2005; Mondadori et al., 2005). After washing in PBS, small antral follicles (1–4 mm at the ovary surface) and medium antral follicles (4–8 mm at the ovary surface) were aspirated with a 5 ml syringe using a 19-gauge needle. Follicular contents were transferred to petri dishes and observed under a stereomicroscope at 38 °C. Selected cumulus–oocyte complexes had a compact and complete cumulus mass and an uniform or a non-visible ooplasm. These corresponded to category 1 of several authors (Blondin and Sirard, 1995; De Loos et al., 1989; Hazeleger et al., 1995; Hawk and Wall, 1994; Leibfried and First, 1979; Madison et al., 1992; Takagi et al., 1998) and to types I and II of other authors (Hasler et al., 1995). COCs were then washed twice in PBS containing 0.5–1% (v/v) penicillin/streptomycin and 2.5% (v/v) heat-treated newborn calf serum (Sigma). Six COCs of each estrous stage and follicle diameter were taken randomly for the present ultrastructural studies.

2.2. Transmission electron microscopy

For transmission electron microscopy processing of cumulus–oocyte complexes, COCs were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h at 4 °C (Hyttel et al., 1986a). After washing in buffer for 2 h at 4 °C, they were postfixed in 2% OsO_4 in buffer, dehydrated in an ethanol series followed by propylene oxide and embedded in Epon. Ultrathin sections were cut with a diamond knife (Diatome), collected on copper grids (Taab), stained with alcoholic (70%) concentrated (1 g/ml) uranyl acetate (20 min) and Reynolds lead citrate (10 min), and observed at 60 kV in a JEOL 100 CXII transmission electron microscope.

2.3. Cytochemistry and immunocytochemistry

Glycogen and glycoproteins were ultrastructurally labelled using the periodic acid–thiocarbohydrazide–silver proteinate technique (Thiéry, 1967).

For chondroitin sulfate detection, ultrathin sections were collected in gold grids and processed in a dark humid chamber (100 μl drops). For removing OsO_4, sections were pretreated in a saturated (1 g/ml) sodium metaperiodate