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Morphologic characterization of isolated bovine early preantral follicles during short-term individual *in vitro* culture

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ABSTRACT

To provide new insights in the molecular mechanism controlling preantral follicular development and to unravel the needs to support *in vitro* follicular development of early-stage preantral follicles (PAFs), there is a need for alternative *in vitro* bovine follicle culture methods. In this study, we aimed to characterize follicular dynamics using an IVC system of isolated and individually cultured bovine early PAFs during 10 days to generate individual follicle follow-up data. Preantral follicles (<50 μm) were isolated from slaughterhouse ovaries and cultured individually for 10 days. Individual follicle morphology, growth, survival, quality, and cell proliferation were evaluated in time by combining noninvasive and invasive assessment methods. The PAFs were light microscopically evaluated during culture to assess follicular dynamics, stained with neutral red to determine follicle viability, stained with 4′,6-diamidino-2-phenylindole and terminal deoxynucleotidyl transferase dUTP nick end labeling to evaluate cell proliferation and follicle quality, and processed for histologic evaluation to assess follicle morphology. On the basis of their morphology, follicles were subdivided in three categories, with category 1 follicles showing the best morphologic features. On Day 0, only category 1 follicles were selected, but follicle categories were reassigned on evaluation Days 1, 2, 4, 7, or 10. Although 67% of the follicles survived 10 days of IVC, the number of follicles exhibiting a normal morphology decreased significantly from Day 7 onward and the apoptotic index increased significantly from Day 10. Both category 1 and 2 follicles showed a significant increase in follicular diameter (Day 10: 21.80 ± 0.86 and 11.82 ± 0.80, respectively). This increase in follicular diameter showed to be correlated with an increase in the total cell number. In conclusion, this culture system showed to support follicular development until Day 10, although the proportion of follicles showing normal morphologic features and the follicular quality decreased after 10 days of IVC. Follicles maintaining their category 1 morphologic features over time seem to be of a better quality and show a higher developmental competence as compared to category 2 and 3 follicles.

1. Introduction

Patient survival rates for most cancer types have improved substantially over the past decades [1–5]. This success can mostly be attributed to a faster diagnosis and

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improved treatments. At present, female cancer patients are given the option to have their ovarian tissue cryopreserved before undergoing ototoxic chemotherapy or radiotherapy. Indeed, mammalian ovaries contain a large number of quiescent immature follicles, also known as primordial follicles. However, the number of primordial follicles is still believed to be limited in most species, despite several reports on oogenesis after birth in mice and prosimian primates [6–9]. *In vivo*, from the first ovulatory cycle until menopause, only a small fraction of primordial follicles will ultimately reach the preovulatory stage, whereas the remaining follicles will degrade by atresia [10]. Although cryopreservation of early preantral ovarian follicles *in situ* can play a crucial role in restoring female fertility after a successful radiotherapy and/or chemotherapy [11–15], subsequent ovarian tissue autotransplantation bears the risk of reintroducing cancer cells [16]. Therefore, there is an urgent need for alternative *in vitro* follicle culture methods, immediately after retrieval or after thawing of cryopreserved follicles.

Because of ethical and economic reasons, the access to human or primate ovarian tissue for research is limited. The use of ovarian tissue from animal species as a model to optimize *in vitro* follicle cultures can therefore be a valuable alternative. On the basis of similarities in anatomy of the ovaries, follicular physiology, and oocyte features, the bovine model is increasingly suggested and accepted as a relevant model for preimplantation female fertility studies [17–19]. Nevertheless, at this moment, it is still impossible to culture bovine primordial follicles *in vitro* until the preovulatory stage delivering a competent oocyte, capable of being fertilized and establishing embryonic development.

Studying bovine *in vitro* follicle culture systems could be of help to further unravel regulatory mechanisms of primordial follicle activation and development. The IVC of preantral follicles (PAFs) embedded in small cortical fragments is by far the most applied culture method [20–24]. In a culture system containing insulin, primordial follicles are activated spontaneously within 2 days after removal from their natural environment [20]. Whether this is the result of removing the primordial follicles from their inhibitory natural environment, established by the hypothalamo–pituitary axis, or mainly caused by the enriched microenvironment of the IVC medium is still a topic for debate. Follicle growth and stage transition were reported, but only a few follicles developed until the secondary stage [20,24]. Although, after isolation from the cortical fragments, these secondary follicles can be individually cultured *in vitro* until an antral-like stage [23,25,26], no complete follicle or oocyte maturation could be achieved so far. However, as cortical fragments contain a large number of PAFs at different developmental stages, individual follicle follow-up during the entire culture period can be challenging [24].

Therefore, there is a growing interest in IVCs for single follicles. This way, follicles of one particular developmental stage can be cultured without the influence of surrounding follicles and stromal cells, and follow-up data can be generated by individually tracking the follicles. Additionally, this could provide new insights in the molecular mechanism controlling preantral follicular development and report-specific needs for support of *in vitro* follicular development of early-stage PAFs. In the past, bovine-isolated early PAFs have been cultured in (small) groups [27–29] to evaluate *in vitro* follicle development and to assess follicle survival after cryopreservation [30]. However, individual PAF dynamics have not yet been fully characterized and survival rates determined during a longer culture period.

Therefore we aimed to (1) characterize follicular dynamics using an IVC system of isolated and individually cultured bovine early PAFs during 10 days, (2) generate individual follicle follow-up data, (3) evaluate individual follicle morphology, growth, survival, cell proliferation, and quality over time by combining noninvasive and invasive assessment methods.

2. Materials and methods

2.1. Isolation and individual IVC of early preantral follicles

All chemicals were obtained from Sigma Chemicals (Diegem, Belgium), unless otherwise noted. Bovine ovaries (n = 5 ovaries per replicate; 3 replicates) were collected at slaughter and transported in warm saline (approximately 25 °C) to the laboratory within 3 hours. On arrival, they were rinsed with 70% alcohol. Small cortical tissue strips (±1 mm^3) were cut and removed using a scalpel and placed in dissection medium (Leibovitz medium; Invitrogen Ltd, Ghent, Belgium) supplemented with sodium pyruvate (2 mM), glutamine (2 mM; Invitrogen Ltd.), BSA (3 mg/mL), penicillin G (100 U/mL), and streptomycin (100 μg/mL). The cortex pieces were mechanically dispersed by an Ultra-Turrax T18 Basic device (IKA; VWR, Leuven, Belgium) and a plastic disperser tool (IKA). The cell suspension was subsequently filtered through a 100-, 60- and 25-μm cell strainer (Greiner, Vilvoorde, Belgium). Early PAFs were recovered from the 25-μm mesh filter by rinsing it with dissection medium. Follicles were visualized using standard inverted light microscopy (Olympus, Aartselaar, Belgium). Preantral follicles with a diameter between 25 and 50 μm, enclosing an immature oocyte surrounded by one layer of flattened or cuboidal granulosa cells and intact basal membrane, were selected [22]. They were individually transferred and cultured in 70 μL of culture medium in 96-well plates (Greiner) at 38.5 °C and 5% CO_2. The culture medium consisted of equal parts of Dulbecco's Modified Eagle's medium and Ham's F12 nutrient supplemented with penicillin G (240 U/mL) and streptomycin (240 μg/mL), fungizone (5 μg/mL), fetal calf serum (2.3%) and newborn calf serum (2.3%), BSA (0.75%), insulin (5 μg/mL), transferrin (5 μg/mL), and selenium (5 ng/mL). The experimental setup is described in the following and summarized in a flow chart (Fig. 1). Per plate, 30 wells were used for culture. On each day of evaluation (0, 1, 2, 4, 7, and 10), the morphology of all individual follicles was assessed and their diameter was measured. Subsequently, one plate per evaluation day and replicate was sacrificed to evaluate follicle viability by neutral red (NR) staining and histologic assessment (60 follicles per evaluation day, divided over three replicates) and to determine follicle quality by the