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Characterization of folliculogenesis and the occurrence of apoptosis in the development of the bovine fetal ovary

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ABSTRACT

The aim of this research was to perform *in situ* quantification, morphometry evaluation, and apoptosis analysis of ovarian follicular wall cells in mechanically isolated follicles obtained from ovaries of bovine fetuses (*Bos taurus indicus*) between 3 and 9 months of age. Apoptosis was evaluated using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay. The number of isolated follicles increased from 3 months onward (102.5 ± 141.1, mean ± SEM), peaked at 6 months (12855.0 ± 9030.1), and then decreased by 7 months (3208.7 ± 3249.5), consistent with atresia occurring at these stages. Follicular density was greatest at 4 months, consistent with a sudden boost in follicular activity independent of a corresponding increase in ovarian size. Antral follicles were first observed at 5 months. As fetal age increased, there was a tendency for the percentage of primordial and primary follicles to decrease, and the percentage of secondary follicles to increase. However, the high variability ($P < 0.05$) for all follicle populations up to 5 months of age precluded further interpretation of these results. Oocyte diameter increased from the primordial (23.6 ± 4.4 μm) to the secondary follicular stages (38.0 ± 14.9 μm). Apoptosis was observed in ovaries from all fetal ages analyzed. We concluded that preantral follicles could be isolated from bovine fetuses by 3 months of age, with apoptosis affecting ovarian follicular dynamics throughout fetal life.

1. Introduction

The female of all domestic species and primates have a finite stock of germ cells that is established in fetal life and then used during folliculogenesis. Despite a large initial follicle pool, only approximately 1% of this population reaches the preovulatory stage during the female reproductive life, with the majority undergoing atresia [1]. For instance, the fetal ovaries of mice, cattle, and humans are initially supplied with an average of 2.5×10^5, 2.1×10^6, and 6.8×10^6 germ cells, respectively [2–4]. Waves of ovarian follicular degeneration have been reported in humans [2] and cattle [3,5], with the bovine ovary containing only approximately 200,000 follicles at birth [6].

Apoptosis, a morphologically distinct form of organized cell death involved in homeostasis of many tissues and organs [7], also has an important role in the processes of ovarian follicular development and degeneration in all mammals. The morphologic hallmarks of apoptosis include cell shrinkage, plasma membrane blebbing, DNA fragmentation, and apoptotic body formation [8]. The ultimate effectors of the apoptotic process are caspases, a group of tightly regulated and highly specific proteases [9]. A cascade of caspase activation resulting from proteolytic cleavage of inactive precursor forms ultimately leads to the activation of caspase 3, the common effector of this process in all tissues [10].

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Apoptosis has been well characterized in follicles undergoing atresia [11–13], with an important role in the physiology and endocrinology of granulosa and theca cells [14,15]. In this regard, the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay has been used to detect apoptosis in many cell types, including in the ovaries of ovine [16] and human [17] fetuses. In the adult bovine ovary, TUNEL and caspase assays have also been used to demonstrate the role of apoptosis in follicles undergoing atresia [18]. Although the ovarian follicular population has been described [19–23], the incidence of apoptosis in the bovine fetal ovary has apparently not been reported.

Several techniques have been developed to isolate oocytes and/or follicles, including mechanical isolation and *in vitro* culture of preantral follicles [24–34]. In this study, we used mechanical isolation of follicles, with the objective of characterizing follicular density, *in situ* morphometry, and the occurrence of apoptosis in ovaries obtained from bovine fetuses of various ages.

2. Materials and methods

2.1. Chemicals and reagents

All products used in this study were purchased from Sigma Aldrich Chemical Company (St. Louis, MO, USA).

2.2. Animals and samples

Bovine fetuses were obtained from dissection of the uterus of cows (*Bos taurus indicus*) after slaughter.

2.3. Experiment I: Mechanical isolation of preantral follicles and determination of follicular density

Ovaries were collected from 38 fetuses between 3 and 7 months of age and used to isolate and quantify the follicular population. Fetal age was determined as follows

$$X\,(X+2) \;=\; Y$$

where X = number of months of gestation; 2 = constant for the cow; and Y = crown-rump length of the fetus (CRL).

The CRL is the crown-rump distance along the vertebral curvature, that is, from the highest point of the head to the first caudal vertebra [35]. After collection, ovaries were weighed on an analytical balance and washed with 70% alcohol and 0.9% saline solution containing antibiotics. Thereafter, ovaries were stored in sterile bottles (containing PBS with 200 IU/mL of penicillin and 200 mg/mL of streptomycin) and transported to the laboratory in isothermal containers at ambient temperature (approximately 30 °C). For each pair of ovaries, one was used for mechanical isolation, and the other was processed histologically to assess follicular density and morphology.

For follicular isolation, ovaries were sliced using a tissue cutter (tissue chopper Mc Ilwain, The Mickle laboratory Engineering Co., Ltd., Goose Green, Guildford, UK) into fragments of 75 μm and placed in TCM-199. Fragments were then mechanically dissociated by repeated pipetting with a 1000 μL automatic pipette and filtered successively through nylon meshes of 500 then 100 μm. Isolated follicles were counted and classified under an inverted microscope.

To determine follicular density (primordial follicles/mm^2), ovaries were fixed in 10% paraformaldehyde, embedded in paraffin, sectioned with a Leika RM 2245 microtome (Leika, Richmond, IL, USA) at a thickness of 7 μm and stained with hematoxylin and eosin. For consistency, data were gathered using the fifth, 15th, and 25th histological sections of each ovary [36]. Quantitative analyses were based on calculating the surface area of the cortical and medullar regions, and then counting the follicles present in each. Surface area measurements were performed using a digital planimeter and AutoCAD (Autodesk Inc., San Rafael, CA, USA).

For *in situ* follicle quantification, ovaries collected from 24 bovine fetuses aged 4 to 9 months, fixed in 10% paraformaldehyde, and transported to the laboratory for histologic processing. After paraffin embedding, 7-μm thick serial sections were made with a microtome and then stained with hematoxylin and eosin as described above. Only primordial, primary, and secondary follicles with an equatorial section yielding a visibly spherical nucleus within the oocyte were used for quantification (total number of follicles), follicle size measurement, and for counting the number of granulosa cells (normal and apoptotic). To determine the follicular and oocyte diameter, an ocular micrometer (Zeiss) coupled to a light microscope (Olympus CH30, Melville, NY, USA) with a 40× objective was used.

The total number of follicles (N) was calculated using the following formula [37]:

$$Total\ N \;=\; \frac{No.\ of\ follicles \;\times\; total\ number\ of\ cuts\ per\ ovary \;\times\; cut\ thickness}{No.\ of\ cuts\ analysed \;\times\; mean\ nucleus\ diameter\ of\ each\ follicle\ class}$$

Quantification of each follicular class (primordial, primary, and secondary) was performed in ovaries grouped by fetal age.

2.4. Analysis of apoptosis using the TUNEL assay

Apoptosis in ovaries from bovine fetuses aged 4, 5, 7, 8, and 9 months was evaluated using the TUNEL assay (TACS XL-BASIC, R & D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Samples were evaluated under light microscopy (Olympus CH30, magnification × 100). Apoptosis was subjectively coded as densely present (+++), moderately present (++), rarely present (+), or absent (−), as described [18], and classified per age group. In addition, the number of TUNEL positive cells was counted for each follicular class (i.e., primordial, primary, and secondary).

2.5. Statistical analysis

Statistical analyses were performed using BioEstat 5.0 software [38] and differences were considered significant at a $P < 0.05$. Linear regression and correlation analyses