ISOLATION AND CHARACTERIZATION OF PREANTRAL FOLLICLES FROM FOETAL BOVINE OVARIES

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SUMMARY

A simple, mechanical method is described for the isolation of preantral follicles from bovine foetuses of 220-280 days of gestation. On average, 2918 + 621 (s.d.) preantral follicles were isolated per ovary. The isolated preantral follicles were characterized on the basis of the morphological appearance of the surrounding granulosa cells, the number of granulosa cell layers, and their diameter. The results show that primordial, primary, and secondary follicles differ morphologically and that they can be classified by their diameter.

INTRODUCTION

Most mammals restrict oogonial proliferation to the prenatal or early postnatal period of development (9). Primordial follicles stay quietly in the ovary for a variable period. The pool of primordial follicles, from which follicles start growing, remains rather constant until the animal is four to six years of age, although variations have been found from 18,000 to 200,000 (prenatal) and zero (sterile) to 700,000 (postnatal) (5, 6). In cattle, primordial follicles are first activated to enter the growth phase of folliculogenesis during foetal life, i.e. at about day 140 p.c. (14).

Information about the factors involved in early folliculogenesis has increased considerably with the isolation and culture of preantral follicles from mice (4, 11, 15), rats (2) and hamsters (13). In vitro, the growth of primary follicles has been shown to be dependent on the presence of adequate concentrations of follicle-stimulating hormone (11) and on the maintenance of metabolic coupling between oocyte and granulosa cells (3). In mice, preantral follicles have been isolated from the ovary and succesfully grown, matured, and fertilized in vitro; live young have been born (4). The factors involved in the initiation and control of early follicle growth in large mammals may differ from those in rodents, as has been demostrated for oocyte maturation (10).

The ability to culture preantral follicles could substantially increase the yield of oocytes available for in vitro fertilization and cloning experiments (11). Additionally, the use of foetal loocytes for in vitro fertilization considerably shortens the generation interval (1). However, before the control of early follicular development in farm animals can be studied, efficient methods for the isolation and characterization of preantral follicles have to be developed. Information about preantral follicles in cattle is limited to histological studies of bovine ovaries (8, 12, 14). To our knowledge there is no information available about the morphological characteristics of bovine preantral follicles after their isolation from the ovary.

The aim of the present study was to develop a method for the isolation of intact preantral follicles from bovine ovaries from foetuses in the final trimester of gestation, and to investigate whether primordial, primary, and secondary follicles can be distinguished, after their isolation from the ovary, on the basis of their morphological appearance and diameter.

MATERIALS AND METHODS

Bovine foetuses (n=8) with a crown-rump length of 70-90 cm, approximately between 220 and 280 days of gestation (14), were collected at a local slaughterhouse and transported to the laboratory within 1 h. Under aseptic conditions, the ovaries were removed, stripped of surrounding fat tissue, and washed in phosphate buffered saline (PBS) supplemented with 5% foetal calf serum (Boehringer Mannheim, Germany), 200 I.U./ml of penicillin and 200 μg/ml of streptomycin (PBS+).

Histology

In order to define size classes for primordial, primary and secondary follicles, two foetal ovaries were fixed in Bouins fluid for 24 h and prepared for histological examination (16). The diameters of oocytes and follicles were measured with an ocular micrometer in a section containing the nucleus of the oocyte. Preantral follicles were characterized according to Höllinger et al. (8), i.e. primordial follicles are oocytes that are surrounded by one layer of squamous or squamous-cuboidal follicle epithelium; primary follicles have a single layer of cuboidal follicle epithelium; and secondary follicles are surrounded by two or more layers of cuboidal epithelial cells.

Isolation of preantral follicles

Each ovary was transferred to a Petri dish with 10 ml PBS+ and dissected into small fragments with watchmakers forceps. The fragments were pipetted into a tube with 10 ml PBS+ and suspended by repeated (50 times) suction-aspiration with a pasteur pipette. After the large tissue fragments had settled, the upper suspension was transferred to a centrifuge tube and centrifuged for 5 min at 70 x g. The supernatant was removed and the pellet was resuspended in 10 ml PBS+.

The suspension was examined in a Petri dish under an Olympus IMT2 inverted microscope. The number of follicles isolated was counted. A total of 9160 follicles, isolated from three ovaries, were classified by diameter and morphological appearance. Follicular diameters were measured at 300x magnification with an ocular micrometer.

To evaluate the classification, isolated follicles were fixed in

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Table 1: Follicle and oocyte diameters measured in 5-μm tissue sections of ovaries from 7- to 9- month old bovine foetuses.

		mean diameter (μm)	
Follicle type	n	oocyte	follicle
Primordial	180	27.9(3.3)[a]	34.6(3.7)[a]
Primary	117	31.6(4.3)[b]	46.1(6.1)[b]
Secondary	61	45.6(14.0)[c]	101.7(41.8)[c]

Standard deviations are given between parentheses
Data with different superscripts in the same column are significantly different.

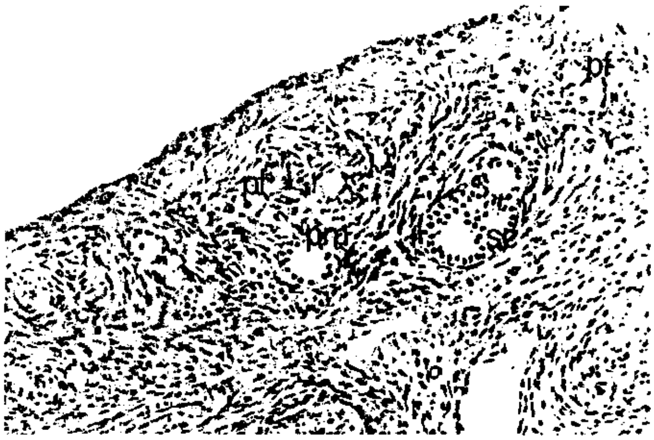

Figure 1. Photograph of a 5-μm section of a foetal bovine ovary. Primordial (pf), primary (pm) and secondary (sc) follicles are located in the cortex of the ovary (x200).

Bouins fluid for 3 h, embedded in 2% AGAR, and prepared for histological examination.

Statistical analysis

Experimental data were analysed with an analysis of variance (Kruskal-Wallis ANOVA and T-test). Values were considered statistically significant when $p<0.01$.

RESULTS

Histology

Numerous primordial, primary, and secondary follicles were observed in the cortex of the foetal bovine ovary (figure 1). In addition, a few antral follicles were found.

The mean diameters of primordial, primary, and secondary follicles and their oocytes, as measured in 5-μm tissue sections, are shown in table 1. There were significant differences in both oocyte diameter and follicle diameter. On the basis of these results, the follicles were divided into three size classes: i) a size class of 30-40 ,μm, containing primordial follicles, ii) a size class between 40 and 60 ,μm, containing primary follicles, and iii) a size class > 60 ,μm, containing secondary follicles.

Follide isolation

On average, 2918 ± 621 (mean number ± s.d.) preantral follicles were isolated per ovary. The isolated follicles varied in size and appearance (figure 2a). Primordial follicles were characterized by a dense, granulated oocyte surrounded by one,layer of flattened granulosa cells (figure 2b). In primary follicles, only a small part of the oocyte was visible; the remainder was covered by one layer of cuboidal granulosa cells (figure 2c). Secondary follicles had more than one layer of cuboidal granulosa cells. The oocyte was not always clearly visible because it was covered by granulosa cells. Occasionally, the granulosa cells were surrounded by one or more layers of flattened cells (figure 2d). Oocytes without or partially surrounded by follicular cells, i.e. denuded oocytes, were also present. Histological evaluation confirmed the classification on the basis, of morphological appearance.

The average diameters of the different types of preantral follicles are shown in figure 3. Isolated primordial, primary, and secondary follicles had a mean diameter of 36.7, 46.6, and 78.0 μm, respectively. The diameters of the different types of follicles were significantly different from each other and were similar to those measured in tissue sections.

Primordial follicles formed 12.4 % of the isolated preantral follicles, whereas primary follicles, secondary follicles, and denuded oocytes formed 57.2 %, 20.7 %, and 9.6 %, respectively. The isolated preantral follicles were classified into the size classes as described in table 1; the results are shown in table 2. The class of follicles of 30-40 μm diameter was composed of primordial follicles (47.8 %), primary follicles (25.9 %), and denuded oocytes (26.3 %). In the class of follicles with a diameter between 40 and 60 μm, 88.8 % of the follicles were primary follicles, the percentage of primordial (3.4 %), secondary (2.7 %), and denuded (5.1 %) follicles being low. Of the follicles with a diameter greater than 60 μm,93.6 % were of the secondary type, 2.1 % were primary and 4.3 % were denuded.

DISCUSSION

This article describes morphological aspects of preantral follicles isolated from foetal bovine ovaries in the third trimester of gestation. Foetuses between 210 and 280 days of gestation were used because at this time primordial follicles are separated from the germ cell cords, large numbers of follicles are present, and follicular development has started (14). Foetal ovaries of this age contain an average of 68,000 ±

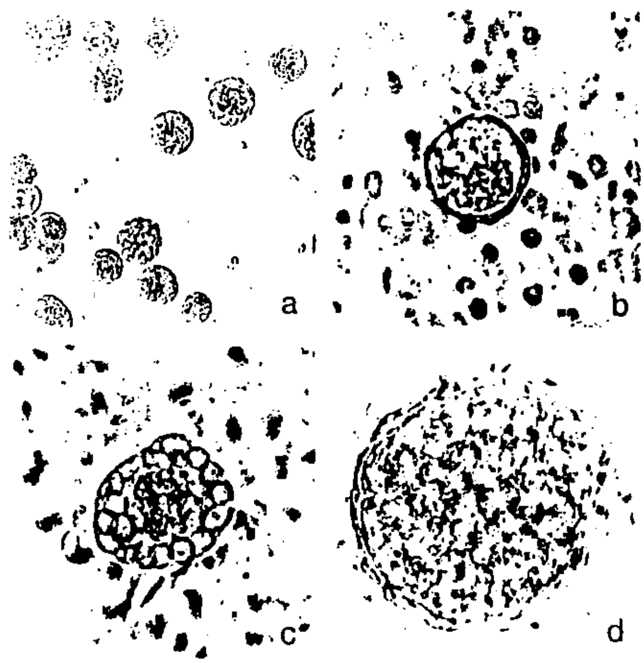

Figure 2. Photographs of isolated preantral follicles. Group (2a, x160), primordial (2b, x480), primary (2c, x 480), and secondary (2d, x480) follicles.

Table 2: The classification of isolated preantral follicles on the basis of morphological appearance and follicular diameter. Number of follicles are indicated.

Size class (diameterr µm)	primordial	primary	secondary	denuded	Total
	morphologiclal appearance				
30-40	960	520	-	530	2010
40-60	180	4680	140	270	5270
> 60	-	40	1760	80	1880
Total	1140	5240	1900	880	9160

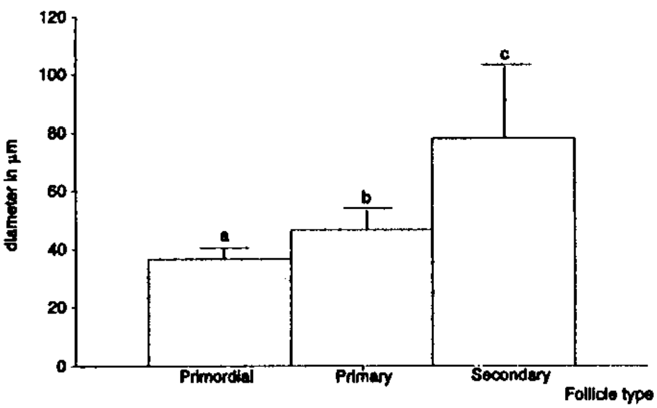

Figure 3. Mean diameters (± s.d.) of primordial (n=114), primary (n=524), and secondary (n=190) follicles after isolation from foetal bovine ovaries. Bars with different superscript are significantly different

21,000 follicles (5).

Approximately 3000 preantral follicles were isolated per ovary. The low number of isolated follicles compared to the number of follicles present in the ovary may be caused by loss in the several steps of the isolation procedure. Loss is quite common in all methods of cell isolation (13). The low number of isolated primordial follicles observed in the present study compared to the number of primary and secondary follicles may be because primordial follicles are tightly embedded in the tunica albuginea (7), whereby mild mechanical treatment is not sufficient to isolate primordial follicles. Primary and secondary follicles can be isolated more easily since they move to the medulla during development (14). Enzymatic treatment of the ovaries may be necessary to isolate primordial follicles. In the pig, Greenwald et al. (7) succeeded in isolating large numbers of primordial follicles (approximately 180.000 per ovary) by using a combined mechanical and enzymatic method.

The average diameters of isolated primordial, primary, and secondary follicles were similar to the diameters measured in tissue sections. The size classes of preantral follicles corresponded with those previously described by Hölliger (8) and Rajakoski (12) with regard to tissue sections from adult cow ovaries. The proposed size classes could be useful as 88.8 % of the primary follicles and 93.6 % of the secondary follicles were found in the classes with diameters between 40-60 µm and > 60 µm, respectively.

In conclusion, preantral follicles from foetal bovine ovaries can be isolated easily and in sufficient numbers for morphological classification. Isolated primordial, primary, and secondary follicles can be distinguished on the basis of morphological criteria. In addition, follicle diameter can be used to distinguish between primary and secondary follicles. Studies on the development of preantral follicles in vitro are in progress.

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