Comparison of Mechanical and Enzymatic Methods for the Isolation of Bovine Ovarian Follicles

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ABSTRACT

The isolation of preantral follicles from the ovaries of bovine was performed under mechanical and enzymatic methods. A significant increase in the total number of follicles retrieved was detected when tissue chopper was used. Micro-dissection could supply good quality, larger sized follicles (400 to 700 μm) but with the lowest yield (9.0±1.0). The isolated preantral and early antral follicles were cultured for 14 days. Follicles isolated by the mechanical method had a greater growth during a culture period than follicles collected enzymatically. Morphologically normal bovine oocytes from early antral follicles after 14 days culture were 59.6% after culture and after 24 h of maturation culture, 12.9% of *in vitro*-grown oocytes reached the second metaphase. In conclusion, this study showed that mechanical method can be used effectively to isolate intact preantral follicles from bovine ovaries.

(Key words : bovine, follicles, isolation)

INTRODUCTION

The bovine ovary contains tens to hundreds of thousands of oocytes (Erickson, 1966). However, during *in vivo* maturation most of these follicles gradually become atretic. This is why only 0.05% of them reach the preovulatory stage (Saumande, 1981). So, most scientists can obtain mature fertilizable oocytes via superovulation or *in vitro* maturation. However, efficiency remains low because their availability is limited by the number of antral follicles present in the ovaries. If bovine oocytes could be harvested in greater numbers from preantral follicles, and be successfully grown *in vitro* to reach meiotic competence (van den Hurk *et al.*, 1997), it would offer a significant means for the propagation of current reproduction technique.

A technique for isolating follicles from rat and mouse ovaries using enzymes has been developed by several groups (Eppig and Schroeder, 1989; Torrance *et al.*, 1989). These methods of the isolation obtain a granulose cell-oocyte complex (GOC). GOC's can be grown *in vitro* (mouse: Eppig and Schroeder, 1989, hamster: Roy and Greenwald, 1989) producing developmentally competent oocytes. Isolation of pig preantral follicles using enzymatic digestion by collagenase has been demonstrated (Greenwald and Moor, 1989). And both two species resulted in the recovery of numerous preantral follicles.

Another isolation process is mechanical method without chemical treatment. Isolation of intact preantral follicles by mechanical method has been used initially in murine species (Nayudu and Osborn, 1992). Using this method, the basement membrane and stromal/thecal tissue are retained in follicles.

We attempted to adapt systems developed in murine species to bovine. The objective of this preliminary work was to develop 1) a technique for extracting preantral follicles from the bovine ovary and 2) a culture system for studying folliculogenesis *in vitro*.

MATERIALS AND METHODS

1. Source of Ovaries

The bovine ovaries from random stages of the oestrous cycle were collected from abattoirs. All ovaries were brought to the laboratory in a thermos containing physiological saline at 30 to 35℃. Ovaries preferably lacking corpora lutea and with antral follicles < 3 mm in diameter were used. After severing blood vessels as close to the hilum as possible, the ovaries were rinsed several times in normal saline (0.154 M-NaCl). In a laminar flow hood, ovaries were rinsed with 70% ethanol. Fig. 1A identified by paraffin block that ovarian cortex is a

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rich source of preantral follicles. Therefore this study used cortical slices to evaluate a number of isolation method.

2. Enzymatic Dissociation of Ovaries

After the wash, the ovaries were cut in half and the medulla were removed using a scalpel blade (no. 21) in dissection medium [Dulbecco's phosphate buffered saline (PBS) supplemented with 20% FBS, penicillin G (75 μg/ml) and streptomycin (50 μg/ml)]. The cortical sections were then minced into small pieces (2~3 mm). The cortical pieces were incubated in 1 mg/ml collagenase 1A (Sigma, St Louis, MO, USA) and 0.2 mg/ml DNase I (Sigma) in Leibovitz medium (Gibco-BRL, Life Technologies Ltd.) for 30 or 60 minutes at 38.5℃.

3. Isolation by Tissue Chopper

The cortical portions of ovaries were put into a tissue chopper (Mickle Laboratory Engineering Co., Gomshal, Surrey, England) adjusted to 500 μm and were cut along the longitudinal, transverse and oblique axes. The ovarian fragments were then placed in Leibovitz medium with 10% FBS and suspended about 15 to 20 times with a large Pasteur pipette followed by a smaller pipette. Successive chopping of the ovaries into pieces and mechanical loosening of small follicles by pipettig was carried out. The resulting suspension was filtered through 500-, 300- and 38-μm nylon mesh filters and the large tissue portions remaining on the 500-μm filter were again transferred to the tissue chopper and the procedure was repeated. The accumulated filtrate was processed as described above.

4. *In Vitro* Growth of Preantral Follicles

Twenty morphologically good follicles were cultured individually in 20 μl droplets covered with oil for 14 days in an incubator under 5% CO_2 at 38.5℃. The culture medium was alpha-minimal essential medium (α-MEM) supplemented with 10% FBS, 0.1 mg/ml sodium pyruvate and FSH (100 mIU/ml). Half of the culture medium was replaced with fresh medium on alternate days.

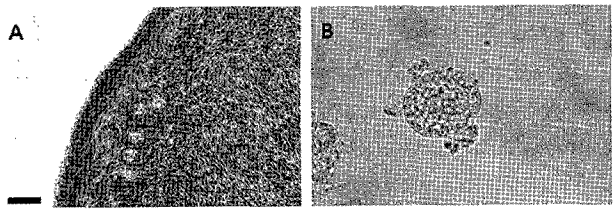

Fig. 1. Preantral follicles from bovine ovary. (A) Histological section, stained with haemotoxylin and eosin, of the cortex of the bovine ovary. Most preantral follicles were located in the tunica albuginea at the periphery of the bovine ovary. (B) Inverted microscopic picture of isolated preantral follicles by using a mechanical method. (Scale bar : 100 μm)

5. *In Vitro* Growth of Early Antral Follicles

From the surface of the ovaries, early antral follicles with 0.4~0.7 mm in diameter were isolated (Fig. 2A). The culture medium was TCM-199 (Gibco-BRL) supplemented with 0.1 mg/ml sodium pyruvate, 0.1 μg/ml estradiol-17β, 4 mM hypoxanthine, and 5% fetal bovine serum (Gibco-BRL, Grand Island, NY, USA). The follicles were cultured on Transwell-COL membrane inserts (3.0 μm pore size, 24.5 mm diameter: Costar, U.S.A.) in six well cluster dishes to prevent the loss of structural integrity between the oocyte and granulosa cells (Eppig and Schroeder, 1989). The follicles were cultured for 14 days at 38.5℃ in 5% CO_2 in air. Half of the medium was changed every 2 days.

6. Collection of Oocytes from Early Antral Follicles and *In Vitro* Maturation (IVM)

After the growth culture, cumulus-oocyte complexes (COCs) were dissected out with fine needles (26G) under a stereo zoom microscope from all the surviving follicles. The COCs (Fig. 2B) with several layers of cumulus cells were selected and washed three times in maturation medium. For *in vitro* maturation, groups of six to eight oocytes were transferred into 50 μl droplets of maturation medium, TCM-199 (Gibco-BRL) supplemented with 10% FBS (Gibco-BRL, Grand Island, NY, USA), 10 μg/ml FSH-P (Folltropin-V, Veterpharm, London, UK), 0.2 mM sodium pyruvate, 1 μg/ml estradiol-17β, and 10 ng/ml EGF. The droplets were then covered with warm (39℃) mineral oil. Oocytes were matured for 24 h at 39℃ in 5% CO_2.

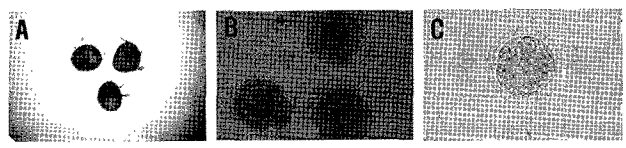

Fig. 2. *In vitro* growth of early antral follicles. (A) Isolated early antral follicles. (B) Cumulus-oocyte complexes (COCs) isolated from a follicle (after culture). (C) Bovine oocytes from early antral follicles after culture and after subsequent IVM for 24 h.

7. Statistical Analysis

Data were subjected to a Generalized Linear Model procedure (PROC-GLM) of the Statistical Analysis System (SAS Institute, Cary, NC, USA). Differences among treatment means were determined by using Duncan's multiple range tests. Statistical significance was established at $p<0.05$.

RESULTS

The isolation of preantral follicles from the ovaries of cows was performed by using either collagenase treatment or a mechanical method using a tissue chopper (Fig. 1B). The effect of different methods of isolating preantral follicles is presented in Table 1. A Significantly higher ($p<0.05$) number of preantral follicles with more viable follicles were obtained with the tissue chopper. Follicular sizes after isolation ranged from 40~140 μm in diameter by enzymatic and mechanical method.

Follicular growth during *in vitro* culture was monitored by measuring the follicle diameter for day 1 to 14 (Fig. 3). Follicles isolated by the mechanical method had a greater growth during a culture period than follicles collected enzymatically. The diameter of the follicles isolated by the mechanical method was increased from day 1 to 8.

Early antral follicles were mechanically isolated from bovine ovaries. Small oocytes, which were contained in early antral follicles and incapable of resuming meiosis, were cultured for 14 days. Of the 52 follicles subjected to growth culture, 31 (59.6%) were morphologically normal and enclosed by cumulus granulose cells after growth culture. Furthermore, 12.9% (4/31) of the oocytes reached M Ⅱ (Fig. 2C) after subsequent maturation culture (Table 2).

Table 1. A comparison of the efficiency of different methods for the collection of bovine follicles

Procedure	No. of ovaries used	Mean no.±SD of retrieved follicles per ovary	Diameter (μm)
Enzymatic isolation (A)	3	40.0±10.9[b]	40~140
Enzymatic isolation (B)	3	12.7± 2.1[c]	40~140
Tissue chopper	3	82.7± 8.3[a]	40~140
Microdissection	3	9.0± 1.0[c]	400~700

[a~c] Mean±S.D.; Values with the different superscripts in the same column are significantly different ($p<0.05$).

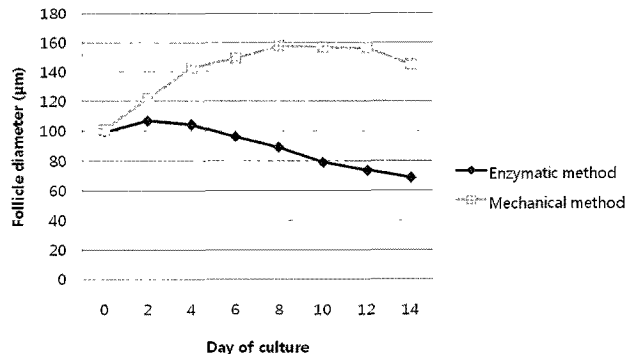

Fig. 3. Change of follicle diameter during 14 days of culture.

Table 2. Nuclear morphology of bovine oocytes grown *in vitro* for 14 days after maturation

Follicular diameter (mm)	No. of follicles		Chromatin configuration (%)		
	Cultured	Matured	GV	GVBD	M Ⅱ
0.4~0.7	52	31	16 (51.6%)	11 (35.4%)	4 (12.9%)

GV : Germinal vesicle stage, GVBD : Germinal vesicle breakdown, M Ⅱ : The second metaphase.

DISCUSSION

In this study, we have evaluated a number of possible methods for the isolation of bovine preantral follicles both by mechanical method and by enzymatic digestion. We have demonstrated that mechanical method yields morphologically normal intact preantral follicles and a significant number of preantral follicles. Preliminary culture studies have shown that the follicles maintain their morphology by 8 days. Current reports demonstrate that preantral follicles fail to grow for more than 8 d in culture.

Histological study have shown that most preantral follicles are found within the ovarian cortex, therefore slices of ovarian cortex were removed from the ovary to evaluate a number of isolation methods.

Enzymatic methods are more expensive to perform and any residual collagenase may affect the ability of isolated follicles to grow and survive *in vitro* (Figueiredo *et al.*, 1993). Consistent with Eppig and O'Vrien (1996), the use of a collagenase treatment lost basement membrane and theca cells during isolation procedure. Also longer digestion times resulted in a fewer number of preantral follicles. These correspond to our demon-

strated in a growth data on bovine preantral follicles. Prolonging the culture period to 14 days decreased the diameter of follicles isolated enzymatic treatment. Since the basal membrane was damaged after isolation, the coupling between oocyte and granulosa cells was apparently weakened. Furthermore, a possible effect of residual collagenase on *in vitro* growth of preantral follicles could be existed.

According to Lucci *et al.* (2002), the use of the tissue chopper proves to be a more powerful method to isolate a great number of preantral follicles without additional enzymatic treatment. Although few follicles were cultured, the present study not only isolated preantral follicles from bovine ovaries, but also grew the diameter of preantral follicles. Our mechanical method may not destroy preantral follicles, which are surrounded by an intact basal membrane.

Isolating early antral follicles by means of micro-dissection produced an average of 9 follicles after 3 h of manipulation. Although micro-dissection has been used successfully to isolate murine large preantral follicles (Nayudu and Osborn, 1992), adaptation of this technique to domestic mammals has been hindered by a larger, more fibrous ovary, making dissection difficult. It is likely that the manual isolation methods cause rupture of intact small follicles. However, micro-dissection was apparently suitable for isolating early antral follicles. Isolation by micro-dissection has been used to release follicles with intact basement membrane (Nayudu and Osborn, 1992). Similarly, we isolated intact follicles and that bovine oocytes isolated from early antral follicles could develop to metaphase II following *in vitro* growth and IVM.

In summary, a number of techniques for the isolation of bovine preantral follicles were examined. The follicular growth rate is a good parameter to compare the effectiveness of different isolation methods. Mechanical method, without the use of enzymatic digestion, yielded morphologically normal intact large preantral follicles which were a suitable starting material for culture. Preliminary studies showed that the follicles maintained their morphology during culture for 8 days. More research is necessary to reach the final goal of establishing a culture system that will support long-term culture and produce meiotically competent oocytes.

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