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In Vitro Growth of Bovine Preantral Follicle under Different Culture Conditions

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ABSTRACT

The objective of this study was to determine effects of different culture media. Preantral follicles were mechanically extracted from bovine ovaries and cultured for 16 days in tissue culture medium (TCM)-199, DMEM or alpha-minimal essential medium (α-MEM) + 10% FBS + 0.1 mg/ml sodium pyruvate + 100 mIU/ml FSH. The collected primary follicles from ovary were higher than the primary and secondary follicles. The survival rates of the follicles in TCM-199 were significantly higher ($p<0.05$) than those in DMEM and α-MEM. The diameter of the follicles progressively increased during 12 days of culture. The maximum size (139.1±5.4 μm) reached on Day 12 of the *in vitro* culture and decreased on Day 16. These results suggest that in a culture of bovine preantral follicles, TCM-199 is an optimal medium and a longer-term culture of preantral follicles (>12 days) may be needed to form antra.

(Key words : Medium, Bovine, Preantral follicle, TCM-199)

INTRODUCTION

At conception, the mamalian ovary contains thousands of small follicles at various stages of development. In the cow, there are on average 130,000 primordial follicles for a given individual. A number of these follicles are activated to enter the growth phase characterized both by the proliferation of granulosa cells and by the increase in the size of the follicles (Gougeon, 2003). 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). If preantral follicles could be efficiently isolated from the ovaries and grown *in vitro* to reach meiotic competence, we would have a great number of potentially viable oocytes of genetic material. For instance, oocytes from preantral follicles would be useful in transgenesis (Betteridge *et al.*, 1989), conservation of endangered species (Johnston *et al.*, 1991) and *in vitro* reproduction techniques (e.g., *in vitro* fertilization, cloning and gene transfer).

The superovulation and *in vitro* maturation are supplied mature fertilizable oocytes. However, efficiency remains low because their availability is limited by the number of antral follicles present in the ovaries. Much work has been undertaken in recent years to produce large quantities of competent oocytes by *in vitro* growth (IVG) systems for preantral follicles. Many culture systems have been used to demonstrate that oocytes grow to full size from preantral follicles in mice (Eppig and Schroeder, 1989; Carroll *et al.*, 1990), rats (Cain *et al.*, 1995) and hamsters (Roy and Greenwald, 1985). Live mouse offspring have resulted from *in vitro* culture of primordial and small preantral follicles (Eppig and Schroeder, 1989; Eppig and O'Brien, 1996). The success in producing meiotic oocytes from large preantral and early antral follicles was acquired in bovines (Telfer *et al.*, 1999). However, in cattle and primates, as well as in humans, very little progress has been made in developing culture systems to grow primordial or primary follicles. In bovines, Wandji *et al.* (1996) developed a culture system for pieces of the ovarian cortex. Most primordial follicles spontaneously activated and developed into primary follicles after 1～2 days of culture, but only a few of the follicles developed into secondary follicles.

Suitable culture conditions are required for *in vitro* growth of bovine ovarian follicles. Several culture media have been used to culture preantral follicles (e.g. mice : alpha modification of Minimal Essential Medium (Nayudu *et al.*, 1992), pigs : North Carolina State University 23 medium (Wu *et al.*, 2001)). The effect of different types of culture media on isolated bovine preantral follicle growth *in vitro* has not yet been analyzed. Therefore, we compared three culture medium

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conditions for their effects on follicular activation and growth *in vitro*. Specifically, we tested the hypotheses that (1) isolated preantral follicles can be grown *in vitro* using an individual follicle culture system and (2) the culture system will activate preantral follicle growth.

MATERIALS AND METHODS

Isolation and Culture of Follicles

Bovine ovaries from random stages of the oestrous cycle were obtained from cows or heifers at a slaughterhouse, placed in PBS at 30～35℃. Only ovaries without a corpus luteum were selected, and each ovary was stripped of surrounding fat tissue and ligaments. The ovaries were rinsed several times in PBS and their surfaces were immediately sterilized with a 70% ethanol solution. Cumulus-oocyte complexes and follicular fluid were removed by the aspiration of ovary antral follicles (2～7 mm diameter) with an 18 gauge needle fixed to a 10 ml disposable syringe before beginning the isolation process. After the aspiration, the ovaries were cut in half and the medulla were removed using a scalpel blade (no. 21) in sterite Leibovitz L-15 medium (Gibco-BRL, Carlsbad, CA, USA). The cortical portions of the ovaries were put into a tissue chopper (Mickle Laboratory Engineering Co., Gomshal, Surrey, England) and were cut along the longitudinal, transverse and oblique axes. The ovarian fragments obtained were placed in Leibovitz L-15 medium and suspended 15 to 20 times with a large Pasteur pipette and than by a smaller Pasteur pipette. The ovarian tissue suspensions were then filtered successively through 500 and 100 μm nylon mesh filters. Each treatment was repeated six times. The nylon mesh filter can remove large ovarian fragments and eliminate the remaining debris. Follicles with an intact basement membrane and even distribution of granulosa and theca layers were selected for culture. The entire process from collection of material from the slaughterhouse to the start of culture was always less than six hours.

Follicles were cultured individually in 96-well culture plates (Biocoat Collagen Ⅰ Cellware, Falcon 354407; Becton Dickinson, France), which were also coated with type Ⅰ collagen matrix. The preantral follicles were cultured for 16 days in 200 μl of a culture medium that had been overlaid with 100 μl sterile mineral oil. All cultures were housed in an incubator maintained at 38.5℃ under a humidified atmosphere of 5% CO_2 and 95% air. The three treatments were: (1) tissue culture medium (TCM)-199; (2) DMEM; (3) α-MEM. The additives used in all the culture systems were 10% FBS (Gibco-BRL, Carlsbad, CA, USA), 0.1 mg/ml sodium pyruvate (Sigma, St. Louis, MO, USA) and 100 mIU/ml follicle stimulating hormone (FSH; Sigma). The day when the follicles were isolated was designated Day 0. To avoid rapid changes in the constituents and pH of the culture medium, half of the culture medium volume was removed and the same amount of fresh medium added to the follicles every other day, starting on Day 2. The follicle diameters were measured at the beginning of the culture period, every other day, by using an ocular micrometer on an inverted microscope. Only follicles with a normal appearance had their diameters measured. The measurements were taken using the average distance between the outer edges of the basement membrane in two perpendicular planes. Follicles were classified as degenerated if they presented one or more of the following aspects: condensed oocyte nucleus, shrunken oocyte, pycnotic bodies in the granulosa cells, low cellular density or basement membrane breakdown (Fig. 2C).

Follicular Histology

A preliminary experiment was conducted to identify follicular appearance. Ovaries collected at a slaughterhouse were sliced (<1 mm thick) with a scalpel blade (no. 21) and fixed overnight in 4% paraformaldehyde solution. Fixed tissues were dehydrated through a graded series of ethanol (70%, 90% and 100%), cleared in xylene, and embedded in paraffin before serial sections (5 μm thick) were prepared.

The serial sections were obtained from the paraffin blocks. The sections were deparaffinized in xylene, rehydrated through a graded series of ethanol (100～50 %), and stained with hematoxylin and eosin (Fisher Scientific, Pittsburgh, PA, USA).

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 using Duncan's multiple range tests. Statistical significance was established at $p<0.05$.

RESULTS

Morphometric Character and Distribution of Isolated Follicles

Histological section showed that primordial follicles had an oocyte surrounded by one layer of squamous granulosa cells; primary follicles had a single layer of cuboidal granulosa cells and secondary follicles had an oocyte surrounded by two or more layers of cuboidal granulosa cells (Fig. 1). The mechanical isolation procedure yielded primordial, primary and secondary fo-

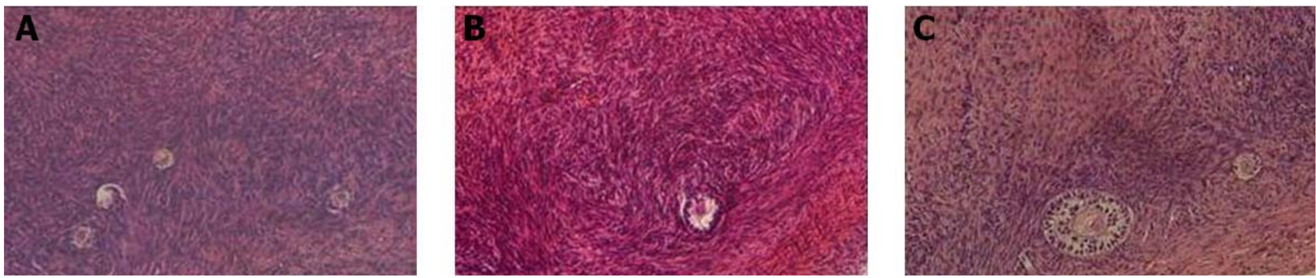

Fig. 1. Histological section of an intact ovary from a bovine. Tissue sections were stained with eosin and hematoxylin. (A) Primordial follicles consist of an oocyte surrounded by a single layer of flattened granulosa cells. (B) Primary follicles are identified by one layer of cuboidal granulosa cells around the oocyte. (C) Secondary follicles are identified by having more than two layers of cuboidal granulosa cells. Magnification is approximately 400× on an inverted microscope.

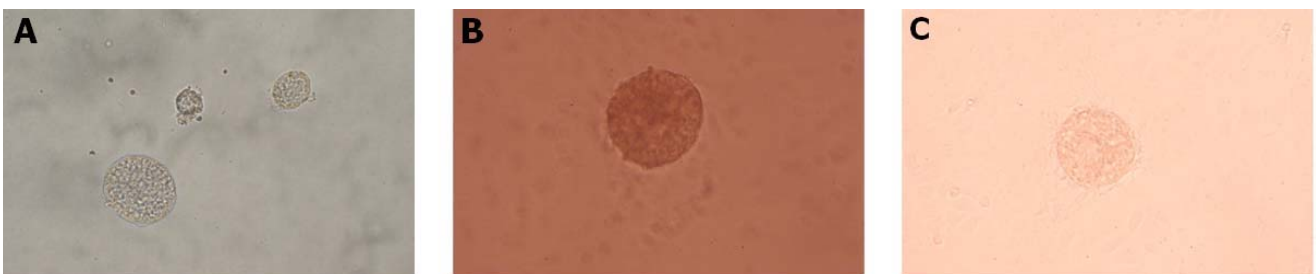

Fig. 2. Inverted microscopic picture of bovine preantral follicles using a mechanical method. (A) Freshly isolated preantral follicle on the start day. (B) Preantral follicle on Day 16 of culture. (C) Degenerated bovine preantral follicles after culture. Magnification is approximately 400X on an inverted microscope.

llicles (Fig. 2A). The distribution of follicles of each category is elicited from histological analysis (Fig. 1). The mean diameters of primordial, primary and secondary follicles isolated were 38.0±0.8 μm, 49.6±1.4 μm and 89.2±2.5 μm, respectively. As shown in Fig. 3, about 56% of the preantral follicles were at the primary follicle stage, while about 5% and 39% were at primordial and secondary follicle stages, respectively.

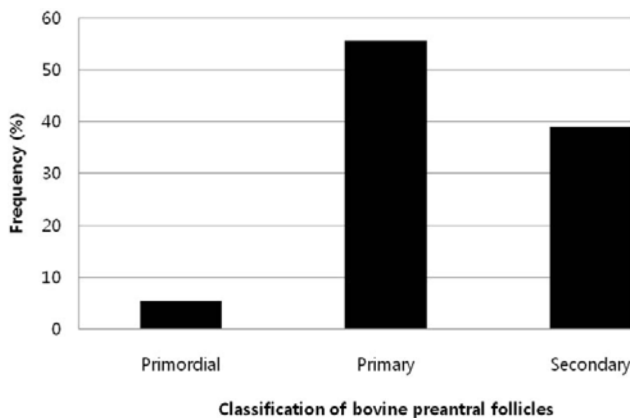

Fig. 3. Overall proportion of primordial, primary, and secondary follicles mechanically isolated from the bovine ovary (n=612).

Effects of Culture Medium on Follicular Survival and Growth

In order to determine the optimal culture media, the bovine preantral follicles were cultured in the three different culture media. With respect to the survival rates of the preantral follicles, there was no degeneration among those cultured with TCM-199, DMEM and α-MEM on Day 4 (Fig. 4). Survival rates decreased during the 16-day culture period.

As shown in Fig. 4, the survival rates of the preantral follicles after 16 days were significantly higher in TCM-199 (82.3%) than in DMEM (42.1%) and α-MEM (53.1%).

The change in follicle diameter during the culture period is summarized in Table 1. 225 follicles were observed in each treatment group and their morphology appeared to be normal during the 16-day periods (Fig. 2B). The mean follicle diameter at the beginning of the culture was 90.2±2.6 μm, 84.2±3.8 μm and 91.6±3.0 μm. During the culture period, the diameter of the follicles increased progressively for the first 12 days. No statistical differences were shown in the 0~4 days of culture among the 3 culture groups. Prolonging the culture period to 16 days decreased the diameter of the follicles in the 3 culture groups.

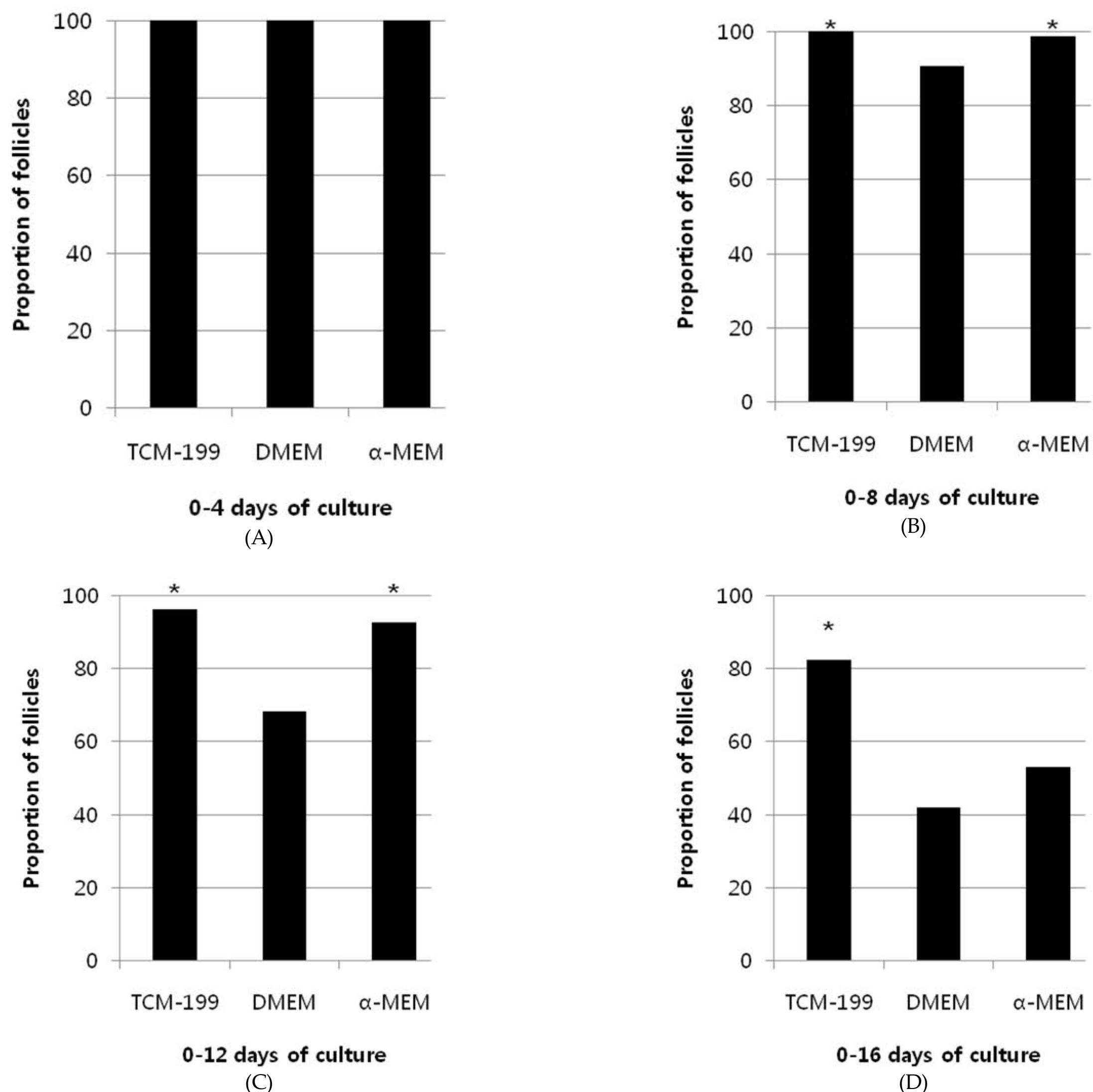

Fig. 4. Survival rates of bovine morphological normal follicles during a 16-day culture period in the three different culture media.
* Significant difference between the three medium groups within days ($p<0.05$). (A) The existence rates of preantral follicles isolated from a bovine ovary grown *in vitro* for 4 days. (B) The existence rates of preantral follicles isolated from a bovine ovary grown *in vitro* for 8 days. (C) The existence rates of preantral follicles isolated from a bovine ovary grown *in vitro* for 12 days. (D) The existence rates of preantral follicles isolated from a bovine ovary grown *in vitro* for 16 days.

DISCUSSION

The development of techniques for the isolation of ovarian follicles is of vital importance to obtain a large yield of follicles for further *in vitro* culture studies (Telfer, 1996). Isolation of preantral follicles from mouse ovaries is relatively easy. Especially in domestic animals, however, the follicle isolation technique is difficult because of the anatomical structure of the ovary. The ovary consists of dense interstitial tissue (Smitz and Cortvrindt, 2001) including a fibrous matrix. And the basement membrane of follicles is surrounded by tightly packed stromal cells (Katske and Rynska, 1998). As shown in Table 1, the majority of preantral follicles retrieved were at the primary stage. In addition to this study, Schotanus *et al.* (1997) reported that about 20% of the retrieved follicles were primordial, and more than 80% of the follicles were at the primary stage. Choi *et al.* (2006) reported that more than half of the retrieved follicles were at the primordial stage. This difference between the two might result from the different status of donor animals including age and reproductive performance. It is well-known that primordial follicles disappear as age increase (Erickson, 1966), and because of the incomplete isolation method

Table 1. Follicle diameter depending on the duration of the *in vitro* culture

Treatment	Follicle (n)	Follicle diameter ± SEM (μm)				
		Day 0	Day 4	Day 8	Day 12	Day 16
TCM-199	95	90.2±2.6[a]	110.9±4.3[a]	133.3±4.3[a]	139.1±5.4[a]	127.9±5.5[a]
DMEM	62	84.2±3.8[a]	97.3±7.3[a]	118.1±7.3[a]	119.6±7.1[ab]	103.3±9.0[b]
α-MEM	73	91.6±3.0[a]	109.5±4.7[a]	127.7±4.7[a]	121.6±6.1[b]	105.7±6.5[b]

Different lower case letters in the same row (a-b) indicate a significant difference within days(p<0.05).

used, many primordial follicles in the ovarian cortex might have been lost during follicle retrieval.

According to O'Brien *et al.* (2003), the choice of culture medium has a major impact on the results of oocyte growth *in vitro*. Likewise, follicle culture studies on the effects of other basal media are necessary. The preantral follicles were grown in various media, such as α-MEM (Nayudu and Osborn, 1992; Cortvrindt *et al.*, 1996), Dulbecco's modified Eagle's media (DMEM) (Liu *et al.*, 1998) and TCM-199 (Christmann *et al.*, 1994; Harada *et al.*, 1997). In the present study, we found that the culture of follicles in the TCM-199 significantly reduced follicular degeneration in comparison with follicles cultured in the α-MEM and D-MEM media. In rats, Kim *et al.* (2008) reported that α-MEM was a better culture medium for growth of mouse preantral follicles than TCM-199. Mao *et al.* (2002), in a study of the porcine preantral follicular culture system, reported that preantral follicles cultured in TCM-199 grew faster compared with the North Carolina State University medium 23 (NCSU23). α-MEM includes precursors of DNA and is suitable for rapidly dividing cell types and these may have beneficial effects on the early follicle stage of mouse development. TCM-199 is a complex medium containing amino acids, vitamins, ribonucleosides, and deoxyribonucleosides in addition to the usual inorganic salts and energy sources (glucose) of a simple medium. So, when follicles were cultured in TCM-199, the survival and growth of culture follicles were improved in bovine and porcine specimens.

The development of a culture system that acquires a meiotically competent oocyte from a preantral follicle in the bovine is an attractive one. The span of follicle development is lengthy in the bovine when compared with the period in the mouse. The transition time from primordial to primary follicle is estimated to be 120 days in humans (Gougeon, 1986). The development from primordial to preovulatory follicle is a time-consuming event, estimated at 180 days in the cow (Lussier *et al.*, 1987). This means that the culture of small preantral follicles will require months to reach full maturity. From our experimental results, the follicle diameter of bovine preantral follicles rapidly increased during the first week of culture and the growth rate was slowed after 8～12 days of culture. And reduction of follicle diameter was noticeable during the last culture period in TCM-199, DMEM and α-MEM, implying that these culture conditions were not ideal to sustain long-term follicular culture.

Although we did not produce an antral follicle with antrum-like structures, the results of the present study provided promising clues to the possible growth of isolated bovine preantral follicles. More research is necessary to reach the final goal of establishing a culture system that will support long-term cultures and produce meiotically competent oocytes.

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