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Morphological Criteria of Bovine Ovaries for Predicting Retrieval Efficiency of Preantral Follicles

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ABSTRACT : To predict the number of preantral (primordial, primary and secondary) follicles retrieved from bovine ovaries, we examined the relationship between morphological parameters of ovaries and number of preantral follicles retrieved mechanically. The preantral follicles were retrieved mechanically by slicing ovarian tissue and the influences of size of the ovaries, number of antral follicles, and presence of cystic follicle and corpus luteum on the retrieval were evaluated. Total 77 ovaries were used and significant ($p<0.05$) relationship was detected between the number of antral follicles and the presence of cystic follicles, and the retrieval number. More preantral follicles were retrieved from the ovaries having more than 20 antral follicles than those having less than 20 antral follicles (17,760±5,637 vs. 3,689±537) in the ovarian cortex. The retrieval number was significantly reduced in cystic ovaries compared with non-cystic ovaries (5,167±825 vs. 20,631±6,507). However, neither ovary size (<3.5, 3.5 to 4.0, 4.0 to 4.5 and >4.5 cm) nor the presence of corpus luteum affected the follicle retrieval. In conclusion, the number of preantral follicles retrieved from the ovaries can simply be predicted by the number of antral follicles and the presence of cystic follicles in the ovarian cortex. (**Key Words :** Bovine, Ovary, Preantral and Antral Follicle, Retrieval, Atresia)

INTRODUCTION

Retrieval of oocytes is important for undertaking novel reproductive biotechnologies in current animal sciences. In mammals, less than 1% of ovarian follicles only develop into Graafian follicles where intrafollicular oocytes meet a chance to mature (Erickson, 1966). To retrieve large number of oocytes, several methods for supporting oocyte maturation in preantral (primordial, primary or secondary) follicles have recently been developed. Eppig and his colleagues (Eppig and O'Brien, 1996; O'Brien et al., 2003) first showed a derivation of developmentally-competent oocytes from preantral follicles by *in vitro*-culture, which yielded live births after embryo transfer. Pivotal roles of follicle stimulating hormone (FSH), epidermal growth factor (EGF), insulin-like growth factor (IGF) and IGF binding protein (IGFBP) in *in vitro*-growth of the preantral follicles have subsequently been reported (Monget et al., 1993; Wandji et al., 1996; Manikkam and Rajamahendran, 1997; Saha et al., 2000). We succeeded a derivation of autologous embryonic stem cells by parthenogenesis of intrafollicular, immature oocytes in mice.

Consequently, we tried to apply this technique for improving reproductive efficiency in cattle. Previous efforts did not greatly enhance developmental competence of immature bovine oocytes in preantral follicles. No report on the success of oocytes to fertilize and further develop into embryos has been made. Slaughterhouse-collected, bovine ovaries of unknown genetic background have generally been provided for artificial reproduction, which hinders in undertaking epidemic approach to optimize the relevant procedure. Since the retrieval procedure is laborious and time-consuming, prediction of the retrieved number of preantral follicles is crucial for optimizing standard protocol of the follicle manipulation.

There was no report on morphological criteria of the ovaries for exact predicting retrieved number of preantral follicles. In this study, we subsequently attempted to determine the relationship between morphological parameters of bovine ovaries and retrieval of preantral follicles. A prospective, randomized approach was made for evaluating whether size of the ovaries, number of antral follicles in ovarian cortex and presence of cystic follicles and corpus luteum affected retrieval number of preantral follicles.

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Table 1. Overall proportion of the number of presumptive primordial, primary and secondary follicles to total number of pre-antral follicles retrieved by tissue slicing of bovine ovaries

Types of follicles presumably classified as	Mean (±SEM, μm) diameters of the follicles categorized	Mean percentage (±SEM) of total number of pre-antral follicles in an ovary
Primordial	26.0±2.5	56.1±8.0
Primary	42.5±3.0	32.3±5.5
Secondary	74.3±6.9	11.5±2.8

MATERIALS AND METHODS

Collection of ovaries and retrieval of preantral follicles

Ovaries were obtained from cows or heifers at a local slaughterhouse and placed in 0.9% (v/v) sodium chloride solution supplemented with penicillin (75 μg/ml) and streptomycin (100 μg/ml) at 30 to 32°C. Before manipulating for the follicle retrieval, the ovaries were classified based on morphological criteria. The categorized ovaries were sliced with surgical blade to 3 mm or less in size in 100×20 mm Falcon™ plastic petridishes (Becton and Dickinson, Lincoln Park, NJ) containing Hepes-buffered tissue culture medium (TCM)-199 (cat no. 31100-027; Gibco BRL, Gland Island, NY) supplemented with 0.17 mg/ml sodium bicarbonate (Sigma-Aldrich Corp, St. Louis, MO), 1% penicillin-streptomycin solution (Sigma-Aldrich) and 0.3% (v/w) BSA (cat no. A4161; Sigma-Aldrich). The sliced tissue fragments were additionally chopped and the chopped tissues were rinsed several times in tissue culture medium (TCM)-199. After rinsing, the cell suspension was diluted to easily observe preantral follicles and then distributed into several 60×15 mm Falcon™ plastic petridishes. For complete retrieval of the follicles, the outside bottom of the petridish was lined with surgical blade at one centimeter intervals. The preantral follicles in the cell suspension were carefully counted under a stereo (SMZ type, Nikkon, Tokyo, Japan) or an inverted (TE-300, Nikkon) microscope.

Experimental design

In experiment 1, the preantral follicles collected mechanically were classified as presumptive primordial, primary and secondary follicles based on their morphological appearance and size (Figueiredo et al., 1994; Hulshof et al., 1994; Lucci et al., 2002). The diameters of retrieved preantral follicles were measured under a stereomicroscope using an ocular micrometer. The population and size of the preantral follicles after retrieval were recorded. In experiments 2-5, the ovaries were categorized by number of antral follicles in ovarian cortex (less or more than 20 follicles), number of cystic follicles (none, 1 or 2), size of the ovary (less than 3.5, 3.5 to 4.0, 4.0 to 4.5 or more than 4.5 cm) and presence or absence of a corpus luteum. Follicles of more than 20 mm in diameter were considered as cystic follicles (McNutt, 1927; Calder et al., 1999). The number of preantral follicles retrieved from the ovaries of each category was counted and provided for statistical analysis.

Statistical analysis

The influence of each parameter on the retrieval number was evaluated by a prospective, randomized study. All experiments were replicated four or more times and the data obtained were subjected to ANOVA of the generalized linear model (PROC-GLM) in a SAS program. When the model effect was statistically significant, each value was compared by the least square method. Significant differences among treatments were determined, where the P value was less than 0.05.

RESULTS

Total 77 ovaries were provided for the experiments and the ovaries that were damaged during collection or had pathological legions were excluded from the experiment. Experimental allocation of the ovaries was randomly made during extended period of time, which prevented uneven distribution of experimental sample into each treatment.

Experiment 1; Proportion and mean diameter of preantral follicles retrieved

As shown in Table 1, 56.1% of preantral follicles collected were at the presumptive primordial follicle stage, while 32.3 and 11.5% were at the presumptive primary and secondary follicle stages, respectively. The morphology of follicles of each category is depicted in Figure 1. Mean diameters of primordial, primary and secondary follicles were 26.0±2.5 μm, 42.5±3.0 μm, and 74.3±6.9 μm, respectively.

Main experiments (2-5); Influence of physiological parameter of the ovary on follicle retrieval

Similar proportions of the preantral follicles developed into each stage were collected throughout the experiments, regardless of the ovary parameters. The ratio of the preantral follicle number allocated into each stage was approximately 0.5-0.55, 0.35-0.4 and 0.05-0.1 for presumptive primordial, primary, secondary follicles, respectively. We then counted the total number of preantral

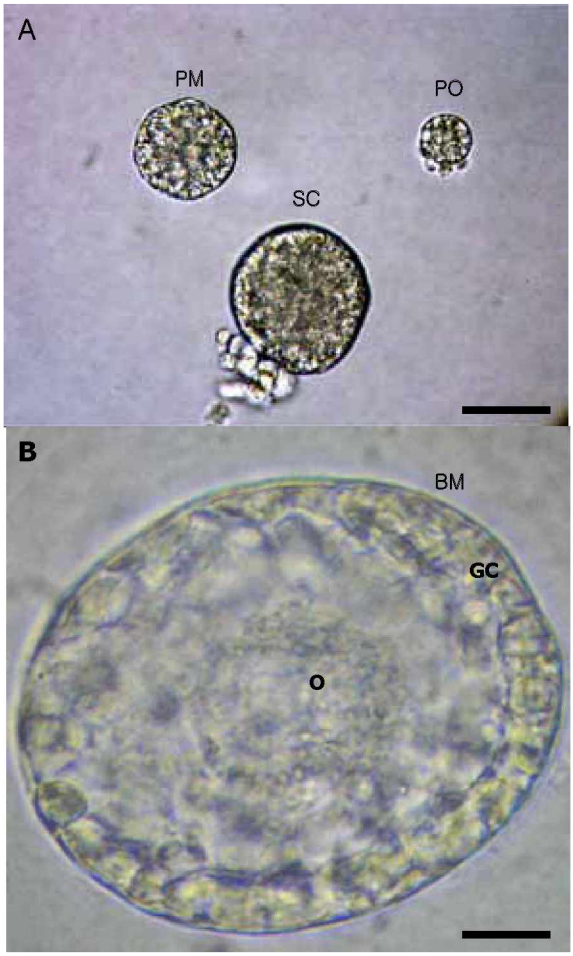

Figure 1. Morphology of preantral follicles retrieved by tissue slicing of bovine ovaries. (A) Preantral follicles of different sizes and morphology were collected, which were presumably classified as primordial (PO), primary (PM) and secondary (SC) follicles (Scale bar; 50 μm). (B) Fresh, mechanically retrieved preantral follicles. Distinct basement membrane (BM), granulosa cells (GC) and oocyte (O) were visible (Scale bar; 10 μm).

(primordial+primary+secondary) follicles for statistical comparison. Number of antral follicles in the ovarian cortex ($p = 0.0273$; Table 2) and presence of cystic follicles in the ovary ($p = 0.0335$; Table 3) significantly affected the retrieval number. More preantral follicles were retrieved from the ovaries containing more than 20 antral follicles than the ovaries containing less than 20 follicles (17,760±5,637 vs. 3,689±537 follicles) in the cortex. As shown in Table 3, the ovaries without cystic follicles yielded more preantral follicles than the ovaries containing 1 or 2 cystic follicles (20,631±6,507 vs. 5,167±825 to 2,950±678 follicles). However, the presence or absence of cystic follicles in the ovary did not significantly ($p = 0.6372$) affect the number of antral follicles in the ovarian cortex. No significant effect was detected in the size of the ovaries ($p = 0.7533$; Table 4) or in the presence or absence of corpus luteum ($p = 0.3022$; Table 5).

Table 2. Relationship between the number of antral follicles in the ovarian cortex and the number of preantral follicles retrieved

Number of superficial follicles in the ovarian cortex	Number of	
	Ovaries examined	Pre-antral follicles retrieved (mean±SEM)
Less than 20	9	3,689±537
More than 20	10	17,760±5,637

Model effect of the categories that was indicated as P value was 0.0273.

Table 3. Relationship between the number of the presence of cystic follicles and the number of antral follicles in the ovarian cortex and preantral follicles retrieved

Number of cystic follicles[a]	Number of		
	Ovaries examined	Antral follicles (mean±SEM)	Pre-antral follicles (mean±SEM)
0	8	25±4.4	20,631±6,507[b]
1	6	19±5.3	5,167±825[c]
2	5	22±6.2	2,950±678[c]

Model effect of the categories that was indicated as P value was 0.6372 and 0.0335 in the number of superficial antral follicles and pre-antral follicles, respectively.

[a] Follicles of more than 20 mm in diameter were considered as cystic follicles.

[b, c] Different superscripts within the same column are significantly different, $p<0.05$.

Table 4. Relationship between the size of the ovary and the number of pre-antral follicles retrieved

Ovary size (cm)	Number of	
	Ovaries examined	Pre-antral follicles retrieved (mean±SEM)
Less than 3.5	5	4,800±1,269
3.5 to 4.0	5	9,160±5,230
4.0 to 4.5	5	13,910±10,131
More than 4.5	5	9,700±2,008

Model effect of the categories that was indicated as P value was 0.7533.

DISCUSSION

The results of this study suggest several morphological criteria of the ovaries for effective retrieval of preantral follicles from slaughterhouse-collected, bovine ovaries: number of antral follicles in ovarian cortex and absence of cystic follicles are important parameters for predicting the retrieved number of preantral follicles. However, there was no relationship between the size of ovary and the presence of corpus luteum, and the retrieval number of the follicles.

As shown in Table 1, the majority of preantral follicles retrieved mechanically were presumably at the primordial stage. In this experiment, the proportion of the number of primordial, primary and secondary follicles was not influenced by the parameter of ovaries. Furthermore, the proportion was not affected by different methods of follicle retrieval (data not shown). Figueiredo et al. (1995) reported in the bovine that more than a half of retrieved follicles

Table 5. Relationship between the presence of corpus luteum in the ovaries and the number of pre-antral follicles retrieved

With (+) or without (-) the presence of corpus luteum	Number of	
	Ovaries Examined	Pre-antral follicles retrieved (mean±SEM)
+	9	14,756±6,211
-	10	7,800±2,717

Model effect of the categories that was indicated as P value was 0.3022.

were at the primary stage. This difference between two studies might result from the different status of donor animals including age, nutritional status, reproductive performance and/or management condition, and even retrieval method. It is well-known that primordial follicles become disappear as the age is increased (Erickson, 1966; Senger, 2003). Lots of primordial follicles in ovarian cortex might be lost during follicle retrieval, which might affect the number of follicles developed into each stage.

The results of this study apparently showed that the retrieval number of preantral follicles was increased as the number of antral follicles in ovarian cortex was increased more than 20. In preliminary experiment, the ovaries were classified into 4 categories by the number of antral follicles in the ovarian cortex (less than 5, 5-10, 10-20 and more than 20 of antral follicles observed in the cortex). Significant increase in the retrieved number of preantral follicles was detected in the ovaries that had more than 20 follicles, while there were no differences in the number among the groups of <5, 5-10 and 10-20. Our results suggest that the ovaries with enhanced activity of folliculogenesis may also have more preantral follicles. This hypothesis is partly supported by our results showing no increase in the proportion of primary or secondary follicles retrieved from the ovaries having large number of antral follicles: number of preantral follicles developed into each stage might proportionally be increased by increasing the number of antral follicles in the ovarian cortex.

The results showing a decreased number of preantral follicles in cystic ovaries (Table 3) may show a relationship between the retrieval of preantral follicles and the regulation of folliculogenesis. Since there was no information on genetic background of the ovaries used in this study, any conclusive interpretation could not be made based on the results of these experiments. Silvia et al. (2002) demonstrated the influence of steroid hormones on follicle growth and the formation of atretic follicle and follicular cysts. Oogenesis, folliculogenesis and follicular atresia are regulated by plenty of signal molecules and growth factors, which are also controlled by steroid or peptide hormone (Richards, 1980; Roche, 1996; Kaipia and Hsueh, 1997; McGee and Hsueh, 2000).

In conclusion, the results of this study suggest effective parameters for predicting population of preantral follicles, which can assist the development of effective method for preantral follicle retrieval. Although more research is necessary to further elucidate the molecular aspects of folliculogenesis and oocyte growth, this research will assist in the development of effective method for preantral follicle retrieval in domestic animals. Derivation of developmentally-competent oocytes can be derived more conveniently if development of gamete biotechnology and innovative artificial reproductive technologies is accompanied with the improvement of the follicle manipulation technique.

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