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Retrieval of Porcine Ovarian Follicles by Different Methods

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ABSTRACT : A series of studies has been conducted to establish a base infrastructure for an ovarian follicle culture system in the porcine and this study was designed to develop an effective retrieval protocol of preantral follicles. Five different methods using collagenase type I (A) or IV (B, C1, C2 and C3), which employed different treatment durations and/or conditions, were employed and sliced ovarian tissue of prepubertal gilts was provided for the retrieval. A significant increase in total number of follicles retrieved was detected when collagenase IV (methods B or C) was used. In total, more ovarian follicles were retrieved by method B undertaking agitation and method C2 without the agitation than method C1 and C3, while the number of preantral follicles collected was the largest in method B. Neither incubation in 5% CO_2 in air atmosphere instead of the agitation nor increased duration of enzymatic treatment up to 120 minutes improved the efficiency of follicle retrieval. There were no differences in the number of follicles retrieved from intact ovaries and from used ovaries for oocyte collection. These results demonstrate the collagenase IV treatment with agitation is effective for retrieving porcine preantral follicles from the ovaries. (**Key Words :** Porcine, Ovarian Follicle, Retrieval, Oocytes)

INTRODUCTION

Studies on porcine reproduction have become important for developing novel biotechnology, as well as for livestock improvement (Denning and Priddle, 2003; Li et al., 2003; Shang et al., 2007; Xu et al., 2007). Producing transgenic pig and establishing pluripotent porcine embryonic stem (ES) cells were major targets of the studies, and securing large number of oocyte is a prerequisite factor for the development. However, there has been a limitation to retrieve sufficient number of the oocytes (Smitz and Cortvrindt, 2001). Numerous efforts have been made to overcome such limitation (Eppig et al., 1989; Eppig et al., 1996; Cortvrindt et al., 1998; Lenie et al., 2004) and also, many of studies have been exerted to optimize oocyte retrieval system from the livestock (Akshey et al., 2005; Yang et al., 2005; Choi et al., 2006). Recently, we developed a culture technique for ovarian follicles in the mouse (Choi et al., 2007; Lee et al., 2007). Mature oocytes derived from the follicle culture have a potential to develop blastocysts and further derive ES cells following subculture of the inner cell mass cells of the parthenotes. Overall, approximately 60% of cultured follicles yield developmentally-competent oocytes and more than 20% of the oocytes developed into blastocysts. This achievement opens a chance to apply the follicle culture system for other species and based on our established technology, we begin to develop a culture system for porcine follicles. To date, no standardized protocol has been suggested for effective retrieving porcine ovarian follicles. The aim of this study is to establish an effective method for retrieving porcine ovarian follicles; we compared several methods using different types of collagenase and treatment protocols, and subsequently evaluated whether the developed method could also be available for the ovaries aspirated for oocyte retrieval, as well as intact ovaries.

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MATERIALS AND METHODS

Collection of ovaries and preparation of ovarian tissues

Ovaries from prepubertal gilts (4 to 6 months of age) were collected at a local slaughterhouse and subsequently transported to the laboratory in a 0.9% NaCl solution maintained 37-39°C within 1 h after collection. The collected ovaries were washed twice in Dulbecco's phosphate buffered saline (DPBS; LB 001-02, WelGENE Inc, South Korea) supplemented with 1% (v/v) penicillin-streptomycin solution (4525, Gibco invitrogen, Grand Island, NY) before follicle isolation. In experiment 2, the ovaries were prepared for follicle aspiration to retrieve oocytes with 18-gauge needle attached to a 5 ml disposable

Table 1. General scheme of enzymatic methods for retrieving porcine ovarian follicles

Methods	With (+) or without (-)		Type of collagenase[b]	Duration of treatment (minutes)
	Agitation	Incubation[a]		
A	+	-	Type I	30
B	+	-	Type IV	30
C1	-	+	Type IV	30
C2	-	+	Type IV	60
C3	-	+	Type IV	120

[a] Performed in a CO_2 incubator maintained at 39°C, 5% in air atmosphere.
[b] Same concentration of collagenase (285 collagenase digestion unit/ml) was used for all treatments.

syringe. After the aspiration, the ovarian cortex of 1 mm in thickness were separated from the whole tissue and the cortex was subsequently cut into 2-5 mm^2 small pieces with surgical blade. After being rinsed several times in DPBS, the tissue was provided for enzymatic treatments.

Enzymatic treatment of ovarian cortex

As shown in Table 1, five protocols for enzymatic digestion were employed. In method A, the sliced ovarian cortex was placed in a 50-ml conical tube (cat. No. 50050; SPL, Korea) containing 10 ml Dulbecco's modified Eagle's medium (DMEM; Gibco Invitrogen) supplemented with 285 collagenase digestion unit (CDU)/ml collagenase type I (C-0130; Sigma-Aldrich Corp, St Louis, MO) and subsequently agitated for 30 minutes with a shaking incubator at 39°C for 30 minutes (Shuttleworth et al., 2002). In method B, the same protocol was employed except that the collagenase type I was replaced with collagenase type IV (C-5138; Sigma-Aldrich Corp). In method C1, C2 and C3, the sliced ovarian cortex was enzymatically digested with collagenase type IV for 30 minutes (C1), 60 minutes (C2) or 120 minutes (C3) without agitation in a CO_2 incubator maintained at 39°C, 5% in air atmosphere.

Collection and classification of isolated follicles

The digested tissues were placed in a 60×15 mm plastic Petri-dish (cat. No. 351007, Falcon, NJ, USA) and rinsed several times in 10 ml DMEM (Gibco Invitrogen) for collecting the follicles. The collected follicles were placed in Dulbecco's PBS supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT). The collected preovulatory follicles were classified into three categories based on their morphology: preantral (100-300 μm), transitional (301-999 μm) and antral (1-3 mm) follicle group (Figure 1). Each classified follicles consisted of multilayer of granulose cells and intrafollicular oocytes. To determine the morphological normality, the criteria of Mao et al. (2002) were employed. The follicle that has spherical shape consisted of the multilayer of granulosa cell layers with well-delineated marginal line and that contains intrafollicular oocyte with evenly-granulated cytoplasm is considered as a normal follicle.

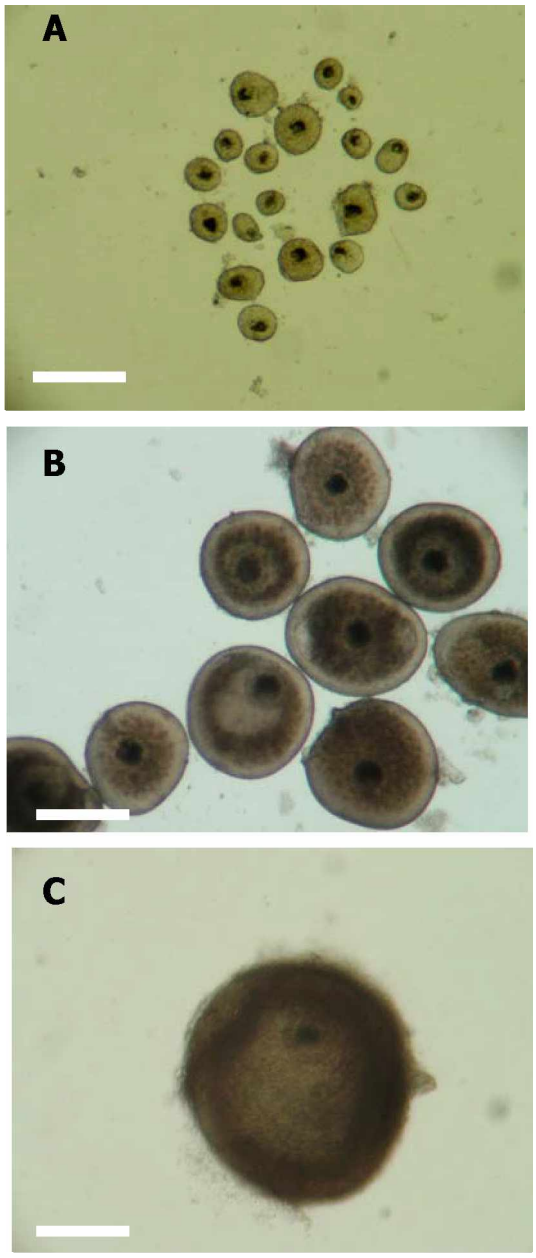

Figure 1. Porcine ovarian follicles retrieved from the ovaries by enzymatic method B. Retrieved follicles were morphologically classified into three categories. (A) preantral follicles of 100-300 μm in diameter and without antrum formation, (B) antrum-forming transitional follicles of 301-999 μm in diameter, and (C) antral follicles of 1-3 mm in diameter. The scale bar is 300 μm.

Experimental design and statistical analysis

In experiment 1, the number of preovulatory (preantal, transitional and antral) follicles developed into each stage was monitored after being retrieved by different enzymatic treatment and morphological normality of the follicles

Table 2. Effect of enzymatic methods on retrieving porcine ovarian follicles

Methods	No. of ovaries used	Total no. of retrieved follicles	No. (%)[a] of follicles retrieved at the stage of			Mean no.±SD of retrieved follicles per ovary
			Preantral (100-300 μm)	Transitional[b] (301-999 μm)	Antral (1-3 mm)	
A	5	412de	185 (44.9)^{c}	216 (52.4)^{c}	11 (2.7)^{c}	82.4±20.9^{de}
B	5	716^{cd}	420 (58.7)^{d}	296 (41.3)^{d}	0 (0)^{d}	143.2±66.7^{cd}
C1	2	57^{e}	19 (33.3)^{ce}	36 (63.2)^{ce}	2 (3.5)^{c}	28.5±4.9^{e}
C2	5	789^{c}	338 (42.8)^{c}	434 (55.0)^{c}	17 (2.2)^{c}	157.8±26.6^{c}
C3	2	75^{e}	19 (25.3)^{e}	54 (72.0)^{e}	2 (2.7)^{cd}	37.5±21.9^{e}

Model effects in the number of totally retrieved, preantral, transitional and antral follicles, and mean number of retrieved follicles per ovary were 0.003, <0.0001, <0.0001, 0.001 and 0.003, respectively.

[a] Percentage of total number of retrieved follicles. [b] The stage between preantral and antral follicle (at the time of antrum formation).

[c, d, e] Different superscripts in the same parameter are significantly different ($p<0.05$).

Table 3. Number of porcine ovarian follicles retrieved from intact or aspirated ovaries

Types of ovary	No. of porcine ovaries provided	Total no. of follicles retrieved[a]	No. of follicles retrieved at the stage of		
			Preantral (100-300 μm)	Transitional (301-999 μm)	Antral (1-3 mm)
Intact	5	772	455	307	10^{b}
Aspirated	5	716	420	296	0^{c}

Model effects in the number of totally retrieved, preantral, transitional, and antral follicles were 0.6616, 0.8113, 0.8891 and 0.0222, respectively.

[a] Method B was used for the follicle retrieval. [b, c] Different superscripts are significantly different ($p<0.05$).

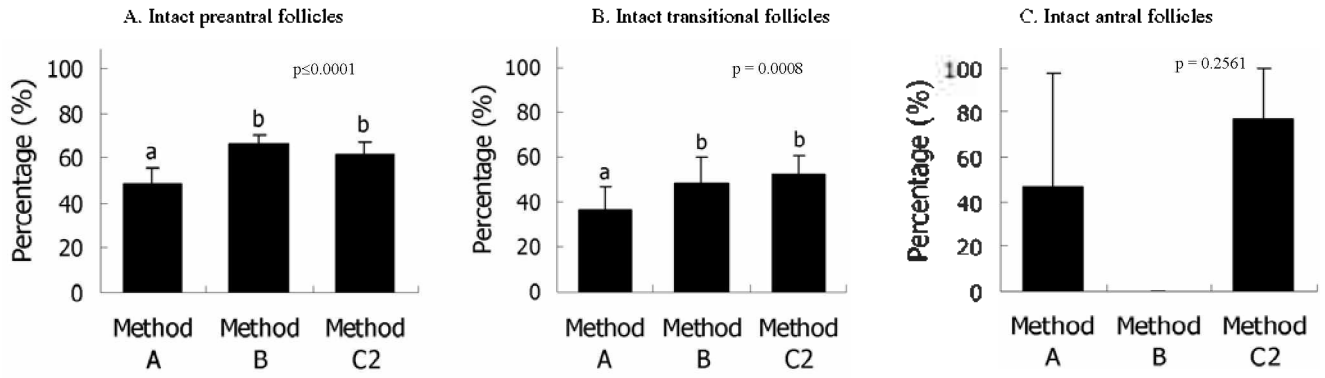

Figure 2. Proportion of morphologically normal (intact) ovarian follicles after being retrieved by different methods. Developmental stage of the retrieved follicles was determined by their diameters and morphology and the diameter was measured by ocular micrometer. More morphologically intact preantral and transitional follicles were retrieved by method B or method C2 than by method A, while there was no significant difference in the antral follicle.

collected was concomitantly counted. In experiment 2, intact ovaries or the ovaries primarily aspirated for antral follicles were provided for enzymatic digestion (method B) using collagenase IV for the follicle retrieval. A prospective, randomized study was conducted and all experiments were replicated five times. The numerical data obtained were subjected to a generalized linear model (PROC-GLM) in a SAS program. When the model effect detected by ANOVA was statistically significant, each value was compared by the least square method. The significant differences among treatments were determined at $p<0.05$.

RESULTS

Comparison of retrieval methods

Overall, total retrieval number of ovarian follicles was larger in the methods B and C2 than the others (716 to 789 follicles vs. 57 to 412 follicles). As shown in Table 2, more follicles were retrieved from one ovary by the method C2 than by the method A, C1 or C3 (157.8 follicles vs. 28.5 to 82.4 follicles). However, the number of preantral follicles retrieved was larger in the methods B than the methods A, C1 and C3, while more transitional follicles were retrieved by the method C2 than the others (434 follicles vs. 36 to 296 follicles). There were few antral follicles retrieved by all methods and the method C2 was the optimal for the retrieval (17 vs. 0 to 11 follicles). The proportion of preantral follicles to total follicles retrieved was larger in the method B than in the methods A and C2, while that of transitional follicles was higher in the methods A and C2 than the method B. The number of preantral follicles less than 100 μm in diameter was not counted in all treatment

groups. More intact preantral or transitional follicles were retrieved by Method B or method C2 than method A, while no significant difference among the groups was detected in the number of intact antral follicle (Figure 2).

Reusing of aspirated ovaries

There was no difference in the number of preantral (420 to 455 follicles) and transitional (296 to 307 follicles) follicles retrieved when the method B was used for collecting ovarian follicles from the sliced tissue of either intact or aspirated ovaries. Several antral follicles however, were retrieved from intact ovaries, while not from the aspirated ovaries (Table 3).

DISCUSSION

Isolation of preantral follicles from mouse ovaries is relatively easy and non-enzymatic protocols have generally been used. Especially in domestic animals, however, the follicle isolation technique is difficult because of anatomical nature of the ovary. The ovary consists of dense interstitial tissue (Smitz and Cortvrindt, 2001) including fibrous matrix, which mechanical isolation cannot be applied without enzymatic digestion (Telfer, 1996). There were several isolation methods applied for retrieving porcine preantral follicles (Donnelly and Telfer, 1994; Hirao et al., 1994; Wu et al., 2001; Mao et al., 2002; Wu et al., 2002), but the efficiency was not promising.

Our finding on the effectiveness of the collagenase on the retrieval of ovarian follicles of different sizes (from 150 μm to 1,000 μm) used was supported by other reports (Morbeck et al., 1994; Telfer, 1996). There seems to be a follicle-specificity in enzymatic treatment and digestion protocol and in this study, collagenase IV was superior to collagenase I for retrieving large number of oocytes without losing morphological normality. It is known that the enzymatic activity of collagenase I is stronger than that of collagenase IV because collagenase I contains additional digestive activities derived from caseinase, clostriapin and tryptic enzyme (McShane et al., 1989). Our results showed that the enzymatic activity of collagenase I would be beyond the optimal for the follicle retrieval. Agitation during enzymatic digestion may further aggravate the efficiency in collagenase I treatment. On the other hand, collagenase IV treatment was much favorable to the retrieval, but the treatment longer than 60 minutes decreased the retrieval efficiency of ovarian follicles. The collagenase IV treatment for 30 minutes may be not so effective as the treatment for 60 minutes. Probably, the efficiency can be improved by the agitation during enzymatic treatment.

The results of experiment 2 showing similar retrieval efficiency between intact and aspirated ovaries by the optimal enzymatic treatment using collagenase IV suggest the feasibility of porcine ovaries for reusing aspirated ovaries to retrieve preantral follicle. This will contribute to securing large number of developmentally-competent oocytes. From different viewpoints, our data suggests that, regardless of physiological nature, there were a large number of preantral follicles remaining in ovarian cortex even after the follicle aspiration. This is independent of anatomical and physiological nature of the ovaries because the porcine is a polyovular species and has a mulberry type ovary containing large number of antral follicles in their cortex.

It is important to evaluate viability of isolated follicles because the objective of this study is to establish culture system for porcine ovarian follicle. Unfortunately, there has been no information on the relationship between the viability of follicular cell and isolated follicle: high survival of follicular cells can not guarantee the development of immature oocytes in the follicles. Instead, we attempted to culture isolated follicles in supplementary experiment and confirmed follicular growth to the pseudoantral stage.

In preliminary study, we retrieved considerable number of follicles of less than 100 μm in diameter. However, we did not culture them successfully because different protocol is necessary for manipulating the small follicles. In fact, we retrieved plenty number of the follicles of less than 100 μm in diameter (at least more than 50 follicles per ovary) and these exceed the number which we can handle. Nevertheless, it is apparent that use of small follicles of less than 100 μm in diameter greatly improves the number of follicles retrieved from one ovary. In the different set of experiments, we are attempting to develop culture system for porcine primary follicles.

In conclusion, enzymatic digestion of porcine ovarian cortex with collagenase is a useful tool for retrieving porcine preantral follicles of different stages. The collagenase IV was most effective. The retrieval efficiency was not affected by follicle aspiration for oocyte retrieval, which was undertaken prior to the enzymatic digestion.

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