

Developmental Competence of Intrafollicular Oocytes Derived from Preantral Follicle Culture with Different Protocols after Parthenogenetic Activation

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ABSTRACT : This study was conducted to improve efficiency of a follicle culture system without reducing developmental competence of intrafollicular oocytes. Preantral follicles (100 to 125 μ m in diameter) of F1 hybrid (B6CBAF1) mice were cultured singly for 216 h in modified α -MEM-glutamax medium, to which 2.5 IU/ml hCG and epidermal growth factor was added 16 h prior to the end of culture. Medium change was either performed three times (54 h interval), twice (72 h interval), once (108 h interval), or not at all (216 h interval). Maturation (progression to the metaphase II stage) of intrafollicular oocytes was detected from 4 days after culture in the three-times change treatment, while all treatments yielded mature oocytes from day 5 of culture. Compared with the three-times change, decreasing the change frequency to once did not reduce the capacity to begin maturation (germinal vesicle breakdown of 82 to 86%), to mature (78 to 79%) and to develop into blastocysts after parthenogenetic activation (29 to 32%). Morphological parameters were similar among these treatments. Except for the no medium change treatment, similar colony-forming activity of inner cell mass cells after culturing of blastocysts in leukemia inhibitory factor-containing medium was detected, while the morphology of the colony-forming cells deteriorated in the change-once treatment compared with the change twice or three-times. In conclusion, the efficiency of the preantral follicle culture system could be improved by reducing frequency of medium change up to a 72 h interval (three times in total 216 h culture) without decreasing developmental competence of oocytes. (**Key Words** : Mouse, Secondary Follicle, Intrafollicular Oocyte, Maturation, Parthenogenesis, Blastocyst Formation)

INTRODUCTION

We recently succeeded to establish homozygous embryonic stem (ES) cell-like cells derived from parthenogenesis of intrafollicular oocytes matured in *in vitro*-cultured preantral follicles. This achievement can contribute not only to securing autologous, immune-specific ES cells without undertaking somatic cell nuclear transfer, but also to retrieving developmentally-competent oocytes from numerous preantral follicles being destined to degenerated. Our effort was subsequently focused to further optimize the culture system and as the series of research, we attempted to increase culture efficiency by decreasing the frequency of medium change. This was because of using single follicle culture system for follicle development, which might have insufficient autocrine or paracrine factors

for oocyte maturation. This study was consequently designed to evaluate whether increasing medium change interval (decreasing the frequency of medium change) could influence developmental competence of intrafollicular oocytes. Morphological parameters including oocyte maturation, development after parthenogenetic activation and colony-formation of inner cell mass (ICM) cells of blastocysts were employed for this attempt.

MATERIALS AND METHODS

Experimental animals

Female F1 hybrid (B6CBAF1; C57BL6 \times CBA/Ca) mice bred in the laboratory of gamete and stem cell biotechnology, Seoul National University were maintained under controlled lighting (14 L:10 D), temperature (20 to 22°C) and humidity (40 to 60%) and two-week-old sexually-immature (prepubertal) females were subsequently provided for this study. All procedures for animal

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management, breeding and surgery followed the standard operation protocols of Seoul National University. Appropriate management of experimental samples, quality control of the laboratory facility and equipment were also conducted.

Isolation of secondary follicles

The females were sacrificed by cervical dislocation and the ovaries were removed aseptically. For mechanical isolation of follicles, the ovaries were placed in 2 ml L-15 Leibovitz-glutamax medium (Gibco Invitrogen, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and 1% (v/v) lyophilized penicillin-streptomycin solution (Gibco Invitrogen) at 37°C. Secondary follicles were retrieved mechanically by using a 30-gauge needle (Lenie et al., 2004).

Culture of secondary follicles

Secondary follicle of 100 to 125 µm in diameter with multiple layers of granulosa cells and an intrafollicular oocyte were collected with an ocular micrometer of an inverted microscope (TE-2000; Nikon, Tokyo, Japan) at 40× magnification. The secondary follicles isolated mechanically from the ovaries were washed three times in 10 µl droplets of L-15 medium and subsequently cultured at 37°C, 5% CO₂ in air atmosphere.

In vitro growth of secondary follicles

The secondary follicles were placed singly in 10 µl culture droplets overlaid with washed-mineral oil (Sigma-Aldrich Corp.) in 60×15 mm Falcon plastic Petri-dishes (Becton Dickinson, Franklin Lakes, NJ). The medium used for the culture of secondary follicle is ribonucleoside and deoxyribonucleoside-containing α-MEM-glutamax medium (Gibco Invitrogen), to which 1% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone), 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 100 mIU/ml recombinant human FSH (Organon, Oss, The Netherlands), 10 mIU/ml LH (cat. no. L-5259, Sigma-Aldrich Corp) and 1% (v/v) penicillin and streptomycin were added. On day 1 of culture, 10 µl of fresh medium was added to each droplet and, from the third day after the replacement, half of a medium was changed. The interval was different according to experimental design.

The maturation of follicular oocytes and parthenogenetic activation

To induce final maturation of intrafollicular oocytes, 2.5 IU/ml hCG (Pregnyl™; Organon) and 5 ng/ml epidermal growth factor (EGF; cat. no E-4127, Sigma-Aldrich Corp) were added to the culture medium 16 hours prior to the end of culture. Retrieved oocytes were freed from cumulus cells

by mechanical pipetting and subsequently placed in M2 medium, consisting of 94.66 mM NaCl, 4.78 mM KCl, 1.71 mM CaCl₂·2H₂O, 1.19 mM KH₂PO₄, 1.19 mM MgSO₄·7H₂O, 4.15 mM NaHCO₃, 20.85 mM HEPES, 23.28 mM sodium lactate, 0.33 mM sodium pyruvate, 5.56 mM vglucose, 1% (v/v) penicillin/streptomycin, and 4 mg bovine serum albumin (BSA)/ml, supplemented with 200 IU/ml hyaluronidase. Initiation of meiotic maturation determined by germinal vesicle breakdown (GVB) and completeness of the maturation to reach the metaphase II stage (having metaphase II plate with polar body) was monitored under a phase-contrast microscope after staining with Lacmoid solution. Size (diameter) of mature oocytes derived from each treatment was also measured by soft imaging system GmbH (Version3.0, Biocompare, Münster, Germany).

Parthenogenetic activation and culture of parthenogenetic oocytes

Mature oocyte freed from cumulus cells were parthenogenetically activated by culturing in calcium-free Potassium Simplex Optimized Medium (KSOM) supplemented with 10 mM SrCl₂ for 4 h. Six to eight oocytes activated were then cultured in a 5 µl droplet of modified Chatot, Ziomek and Bavister (CZB) medium consisted of 81.6 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄·7H₂O, 1.7 mM CaCl₂·2H₂O, 25.1 mM NaHCO₃, 31.3 mM sodium lactate, 0.3 mM sodium pyruvate, 1 mM glutamine, 0.1 mM EDTA, and 5 mg BSA/ml. The oocytes were cultured at 37°C, 5% CO₂ in air atmosphere and development of oocytes to blastocysts were monitored under an inverted microscope (Eclipse TE-3000; Nikon, Tokyo, Japan).

Culture of ICM cells for colony-formation

For culturing ICM cells of blastocysts, zona pellucida of the blastocysts obtained from the parthenogenetic oocytes were removed with acid Tyrode solution and the zone-free blastocysts were cultured on mouse embryonic fibroblast (MEF) feeder layer that were mitotically inactivated with mitomycin C. Knock-out Dulbecco's minimal essential medium (KDMEM; Gibco Invitrogen) supplemented with 0.1 mM β-mercaptoethanol (Gibco Invitrogen), 1% (v/v) nonessential amino acids (Gibco Invitrogen), 2 mM L-glutamine, 1% (v/v) lyophilized mixture of penicillin and streptomycin and 2,000 units/ml mouse leukemia inhibitory factor (LIF; Chemicon, California, US) and 3:1 mixture of FBS and knock-out serum replacement (KSR; Sigma-Aldrich, St Louis, MO) were used for culturing the zona-free blastocysts. Colony-formation of ICM cells was monitored daily under an inverted microscope.

Experimental design

Prospective, randomized study was conducted and all

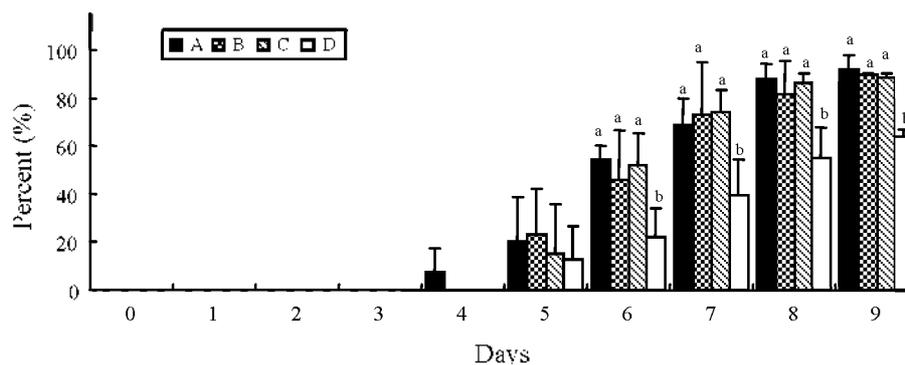


Figure 1. *In vitro*-growth of preantral follicles cultured with different intervals of medium change. Early secondary follicles of 100-125 μ m in diameter that were collected from the ovaries of F1 hybrid mice (B6CBAF1) were cultured in α -MEM-glutamax medium supplemented with fetal bovine serum, insulin, transferrin, selenium and FSH. (A) Change of the culture medium three times within 9 days of total culture duration (54 h interval). (B) Change of medium twice throughout the culture (72 h interval). (C) Change of medium once throughout the culture (108 h interval). (D) no medium change for 216 h. The cultured follicles were monitored daily until the end of the culture under an inverted microscope. A significant ($p < 0.0001$) decrease in the proportion of the follicles reached to the pseudoantral stage was found in the no medium change than other treatments on day 6 to 9 of culture. ^{a, b} Different superscripts within the same day of observation was significantly different.

Table 1. Developmental competence of intrafollicular oocytes derived from *in vitro*-cultured preantral follicles^d

Intervals of medium change ^b	No. of preantral follicles cultured	No. (%) ^c of pseudoantral stage follicles	No. of intrafollicular oocytes developed to				No. (%) ^g of colony-forming inner cell mass cells
			Germinal vesicle breakdown (%) ^d	Metaphase II (%) ^d	Cleaved ^e (%) ^f	Blastocysts ^e (%) ^f	
54	170	134 (79) ^h	110 (82) ^h	67 (50) ^h	44 (66)	20 (30) ^h	4 (13)
72	170	132 (78) ^h	114 (86) ^h	68 (52) ^h	46 (68)	22 (32) ^h	4 (13)
108	170	135 (79) ^h	111 (82) ^h	63 (47) ^h	34 (54)	18 (29) ^h	2 (11)
216	170	94 (55) ⁱ	60 (64) ⁱ	21 (22) ⁱ	14 (67)	2 (10) ⁱ	0 (0)

Model treatment effects in the number of pseudoantral stage follicles, oocytes developed to germinal vesicle breakdown, metaphase II stages, cleaved, blastocysts, and number of colony-forming inner cell mass cells were <0.0001 , 0.0002, <0.0001 , 0.0971, 0.0355 and 0.8246, respectively (indicated as p value).

^a Maturation of oocytes retrieved from the follicles at the pseudoantral stage was triggered by culturing the follicles in the medium supplemented with hCG and epidermal growth factor 16 h prior to the end of follicle culture.

^b (54) Change of the follicle culture medium three times within 9 days of total culture duration (54 h of interval). (72) Change of the medium twice throughout the culture (72 h interval). (108) Change of the medium once throughout the culture (108 h interval). (216) no medium change for 216 h.

^c Percentage of the number of preantral follicles cultured.

^d Percentage of the number of the follicles developed to the pseudoantral stage.

^e Monitored after parthenogenetic activation.

^f Percentage of the number of oocytes developed to the metaphase II stage.

^g Percentage of the number of intrafollicular oocytes developed to blastocysts.

^{h, i} Different superscripts within the same column were significantly different, $p < 0.05$.

secondary follicles retrieved were equally distributed into each experimental treatment. In general, secondary follicles were cultured with medium change three-times (54 h interval; considered as control treatment), twice (72 h interval), once (108 h intervals) throughout the culture (9 days) or not (216 h interval). In experiment 1, hCG and EGF, the final maturation inducers, were added to 16 h prior to the end of day 1 to 9 of culture and meiotic maturation of the oocytes to the metaphase II stage was monitored. In experiment 2, intrafollicular oocytes derived from 9-day cultured preantral follicles were retrieved 16 hours after the treatment with hCG and EGF. Number of follicles grown into the pseudoantral stage, initiation of maturation to reach

the germinal vesicle breakdown and metaphase II stages, meiotic maturation, the morphology of mature oocytes, and cleavage and blastocyst formation after parthenogenetic activation were subsequently monitored. In addition, colony-forming activity of ICM cells of parthenogenetic blastocysts was evaluated after culturing in LIF-containing medium.

Statistical analysis

A generalized linear model (PROC-GLM) in a Statistical Analysis System (SAS) program was employed for statistical analysis. One-way ANOVA followed by the least-square method was conducted when the parameter had

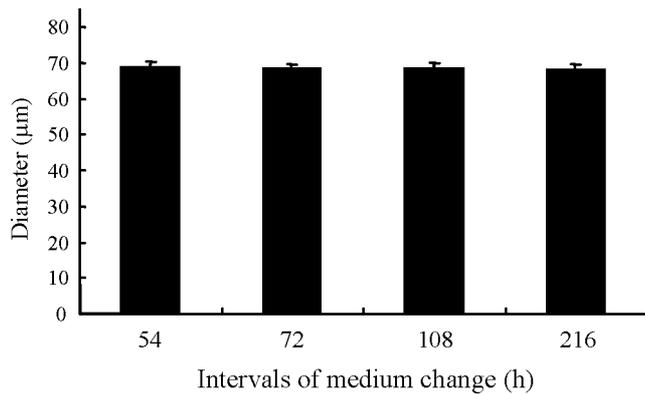


Figure 2. Mean diameter of intrafollicular oocytes developed to the metaphase II stage. The metaphase II stage oocytes were derived from preantral follicle culture with different intervals of medium change; 54, 72, 108 or 216 (no medium change) h interval throughout the culture. No significant difference ($p < 0.3619$) in oocyte diameter was detected among the treatments.

model effect. Significant differences among treatments were determined where the p value was less than 0.05.

RESULTS

Experiment 1

Completion of meiotic maturation was first detected in hCG- and EGF-treated intrafollicular oocytes that were retrieved from preantral follicles cultured for 4 days (Figure 1). Regardless of the treatments, number of mature oocytes were gradually increased and subsequently peaked on day 9 of culture. A significant ($p < 0.05$) model effect among treatments was detected from day 6 to day 9 of culture. Regardless of observation time, more oocytes reached to the

metaphase II stage in the medium change three-times, twice or once (54 to 108 h intervals) than no change (216 h intervals).

Experiment 2

In total, 680 preantral follicles were evenly distributed into 4 groups (170 each). As shown in Table 1, more ($p < 0.05$) follicles developed into the pseudoantral stage in the change three-times, twice or once than no medium change (78 to 79% vs. 55%). Similar pattern was detected in the proportion of oocytes begun maturation (82 to 86% vs. 64%; $p < 0.05$), matured (47 to 52% vs. 22%; $p < 0.05$) and developed into blastocysts (29 to 32% vs. 10%; $p < 0.05$). There was no difference in oocyte morphology among treatments, which were observed by light microscope. Diameters of mature oocytes derived from different treatments were within the range of 68.2 ± 1.6 to 68.8 ± 1.8 μm and no significant ($p > 0.05$) difference was detected among the treatments (Figure 2). In the case of colony-formation of ICM cells, 11 to 13% of ICM cells formed colonies in any groups of medium change, while no colony-formation was detected in no medium change. No prominent difference in the morphology of the colony-forming cells was detected between the cells derived from the change three-times and the change twice (Figure 3). Morphological deterioration, however, was detected in the colony-forming ICM cells derived from the medium change once.

DISCUSSION

The results of this study demonstrated that decreased frequency of medium change to certain extent (increased

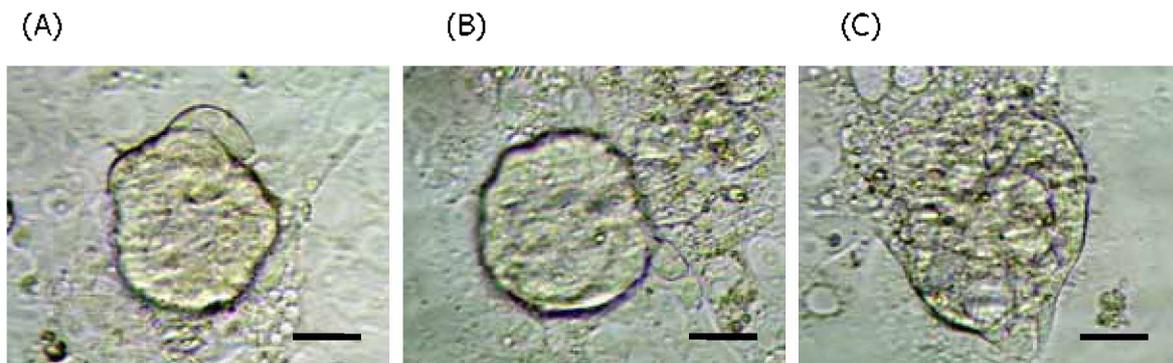


Figure 3. Colony-forming inner cell mass (ICM) cells from blastocysts derived from parthenogenotes of intrafollicular oocytes. Zona-free blastocysts were cultured on feeder layer containing Knock-out Dulbecco's minimal essential medium supplemented with 0.1 mM β -mercaptoethanol (Gibco Invitrogen), 1% (v/v) nonessential amino acids (Gibco Invitrogen), 2 mM L-glutamine, 1% (v/v) lyophilized mixture of penicillin and streptomycin, and 2,000 units mouse LIF (chemicon)/ml and 3:1 mixture of FBS and Knock-out serum replacement were used for initial culture of the blastocysts. (A) Colony-forming ICM cells derived from preantral follicle cultured with 54 h intervals of medium change, (B) Colony-forming ICM cells derived from preantral follicle cultured with 72 h intervals of medium change (C) Colony-forming ICM cells derived from preantral follicle cultured with 108 h interval of medium change. The scale bar = 100 μm .

the change interval up to 72 h) was not detrimental to support developmental competence of intrafollicular oocytes in *in vitro*-cultured preantral follicles. Similar, even insignificantly increased rates of follicle development, initiation and completeness of oocyte maturation, blastocyst formation after parthenogenesis was obtained and there was no change in oocyte morphology by the increased interval of medium change. This modification contributes to improving the efficiency of follicle culture system by reducing labor and maintenance cost without decreasing developmental competence and morphological integrity of intrafollicular oocytes.

In vitro-culture system for preantral follicles is a useful tool for retrieving sufficient number of oocytes for providing various clinical and industrial purposes. Moreover, it also contributes to understanding the underlying mechanisms of oocyte growth and differentiation. Several culture systems for culturing different stages preantral follicles have been suggested in different species (Katska et al., 1998; Cecconi et al., 1999; Wu et al., 2001; Gupta et al., 2002). Live births were derived from *in vitro*-fertilization of intrafollicular oocytes derived from *in vitro*-cultured preantral follicles followed by embryo transfer (Liu et al., 2001; Delapena et al., 2002). On the other hand, homozygous (cloned) animals by transfer of parthenogenetic embryos have been attempted for a long time for efficient undertaking artificial reproduction in domestic animals (Kono, 2006). Recently, homozygous ES cells were derived from parthenogenetic activation of ovulated oocytes in primates including human (Cibelli et al., 2002; Vrana et al., 2004; Sanchez-pernate et al., 2005). We further combined these two technologies for preantral follicle culture and parthenogenetic ES cell establishment, which resulted establishment of homozygous ES cell-like cells (Lee et al., 2006).

Oocytes in preantral follicles cultured *in vitro* were tolerable against reduced frequency of medium change and morphological integrity remained after increasing the change intervals to some extent. In our supplementary data, there was no difference in cortical granule distribution and the formation of chromosome-spindle complex in mature oocytes among treatments (data not shown). Probably, paracrine role of follicular cells such as granulosa and theca cells or autocrine function of intrafollicular oocyte is enough to acquire developmental competence during *in vitro*-culture. In other words, essential substrates for acquiring developmental competence to the blastocyst stage, which were secreted from follicles and/or oocytes, may not be diluted by at least the 108 h interval change.

Data on ICM morphology suggested the deleterious effect of excessive extension of the change interval up to 108 h or 192 h (medium change once or no change during

the 9-day culture) on the colony-formation of ICM cells. These results showed that the 72 h interval (medium change twice during the culture) might be the optimal. Further increased interval might accumulate embryo-toxic metabolites or dried up essential factors for ICM colony formation in medium substrates. On the other hand, it has been reported that the size of intrafollicular oocytes matured *in vitro* was smaller than that of *in vivo*-ovulated oocytes (Eppig and O'Brien, 1996). In this study, we failed to increase the diameter similar to *in vivo*-derived oocytes (around 75 to 80 μm). Additional attempt was subsequently conducted to further improve developmental competence of oocytes, such as applying extracellular matrix gel for three-dimensional culture of the follicles and modifying culture medium composition.

In conclusion, the results obtained from our presented studies contribute to developing alternative technology of immune-specific stem cell establishment (colony-formation rate of more than 10%) and to optimizing reproductive performance of domestic animals having desired genotype. Also, our follicle culture technique could be applied for securing large number of developmentally-competent oocytes from infertile patients with ovarian factor.

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