Effect of Preantral Follicle Isolation Technique on *In Vitro* Follicular Development in Mice

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ABSTRACT

The objective of this study was to compare of different isolation method of mouse preantral follicles, and to examine *in vitro* development of mouse preantral follicles isolated by different method. Preantral follicles were mechanically or enzymatically extracted from mouse ovaries. Mechanical isolation method used fine gauge needles and enzymatic method of isolating follicles used collagenase. The recovered preantral follicles were cultured for 10 days in alpha-minimal essential medium (α-MEM) + 5% FBS + Insulin-Transferrin-Selenium (ITS) + 100 mIU/ml FSH. The collected primary follicles by enzymatic treatment were higher than mechanical method. Others stage preantral follicle by mechanical isolation were higher than enzymatic method. After 10 days of culture, no statistical differences were shown in survival rates of preantral follicle among the 2 culture groups. The metaphase Ⅱ rates of the oocytes were significantly higher ($p<0.05$) in mechanical method (17.8%) than in enzymatic method (5.1%). These results suggest that the isolation method of choice depends on the target stage preantral follicles and mechanical isolation is an optimal method of preantral folliclesin a culture of mouse preantral follicle.

(Key words : mouse, preantral follicle, isolation, *in vitro* culture)

INTRODUCTION

At conception, the mamalian ovary contains thousands of small follicles at various stages of development. 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). The stage of follicle development was defined (Webber *et al*., 2003) as follows: a primordial follicle comprised an oocyte surrounded by a single layer of granulosa cells, of which more than 50% had a flattened, squamous appearance and a primary follicle comprised a single layer of granulose cells, of which more than 50% were cuboidal; secondary follicles were those with more than one layer of granulosa cells, and follicles with more than five layers of granulose cells were designated multilayered preantral. The final stage of follicle development is the tertiary follicle (antral follicle), which is characterized by the formation of an antrum among the granulosa cell layers. However, during *in vivo* maturation most of these preantalfollicles gradually become atretic. This is why only 0.05% of them reach the preovulatory stage (Saumande, 1981). Atresia can occur at any stage of follicular development (Marion *et al*., 1968; Mariana and Nguyyen, 1973). Oocytes derived from antral follicles are commonly used for *in vitro* embryo production (IVP), in spite of the fact that number of preantral follicles in the ovary is much greater than that of antral follicles. If preantral follicles could be efficiently isolated from the ovaries and grown *in vitro* to reach meiotic competence, we would have oocyte bank of genetic material. For instance, a large number of the oocytes in the preantral follicles are potentially useful for an application of embryo transfer, transgenesis (Betteridge*et al*., 1989), conservation of endangered species (Johnston *et al*., 1991).

The earlier aspects of follicular development remain largely unexplored while most of knowledge is restricted to certain aspects of differentiation that occur during the final phases of growth. If isolating preantral follicles and developing them *in vitro* could be successful, detailed understanding of folliculogenesis would be an essential prerequisite for improving the techniques of *in vitro* maturation and fertilization (Driancourt, 1991).

To make use of unused preantral follicles for oocyte production, procedures need to be established for the isolation of

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preantral follicles from ovary. According to Ksiazkiewicz (2006), the success of *in vitro* growth culture of preantral follicles is related to the technique with which the follicles are isolated. Therefore, improvements in efficiency for isolating intact small preantral follicles are needed.

The main aim of the present study was (1) to examine effective isolation of preantral follicles from mouse ovary by mechanical or enzymatic method; (2) to investigate whether both enzymatically and mechanically isolated mouse preantral follicles could survive and maintain their developmental ability.

MATERIALS AND METHODS

1. Isolation of Preantral Follicles

All experiments were conducted using B6CBAF1 (C57BL/6xCBA/Ca) female mice housed and bred in a temperature- and light-controlled room and provided food and water *ad libitum*. For experiments, mice aged 12 days old were killed by cervical dislocation. The ovaries were collected into Leibovitz's L-15 medium (L-15; Gibco BRL, Grand Island, NY, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT).

Mouse preantral follicles were enzymatically (Eppig and Schroeder, 1989) or mechanically (Cortvrindt *et al*., 1996) collected from the ovaries as described previously. Briefly, to isolate preantral follicles enzymatically, the ovaries were immersed into Leibovitz L-15 medium containing 1 mg/ml collagenase (Type 1A; Sigma, St. Louis, MO, USA) and 0.2 mg/ml DNase Ⅰ (Sigma, St. Louis, MO, USA) for 20 min at 37℃ and were triturated by gentle pipetting until the ovaries were dissociated into individual follicles. Mouse preantral follicles were mechanically isolated by using a 30-gauge needle under a stereomicroscope. The selection criteria are as follows: primary follicle of 75 to 99 μm, early secondary follicle of 100 to 125 μm and late secondary follicle of 126 to 150 μm in diameter (Gong and Lim, 2009).

2. *In Vitro* Growth of Preantral Follicles and *In Vitro* Maturation (IVM)

Preantral follicles to be cultured were selected by the following criteria: 1) intact round follicular structure with two to three layers of granulosa cells; 2) the oocyte had to be visible, round and centrally located within the follicle. The follicles were placed in 20 μl culture droplets overlaid with washed mineral oil (Sigma, St. Louis, MO, USA) for 10 days in an incubator under 5% CO_2 at 37℃. The culture medium was a ribonucleoside and deoxyribonucleoside-containing α-MEM-glutamax medium (Gibco BRL, Grand Island, NY, USA) supplemented with 5% (v/v) heat-inactivated FBS, 5 μg/ml insulin, 5 μg/ml transferring and 5 ng/ml selenium liquid medium (ITS; Sigma, St. Louis, MO, USA), 100 mIU/ml FSH and the antibiotics. Half of the medium was changed every 2 days. After 10 days of growth *in vitro*, cumulus-oocyte complexes (COCs) were collected under a stereo zoom microscope from all the surviving follicles. Then follicles were allowed to mature for 16～18 hours in α-MEM supplemented with 1.0 IU/ml human chorionic gonadotrophin (hCG).

The chronological changes in the nuclear status of oocytes after IVM were assessed under a fluorescence microscope after Hoechst 33342 staining. After denudation of cumulus and corona cells by gently pipetting through a mouth-operated micropipette, the oocytes were fixed in 2% formaldehyde in PBS for 10 minutes at room temperature. The oocytes were then placed on slides with a drop of mounting medium consisting of the 3:1 ratio of glycerol versus PBS containing 2.5 mg/ml sodium azide and 2.5 g/ml Hoechst 33342 (Sigma). A cover slip was placed on top of the oocytes, and the edge was sealed with fingernail polish. The stained oocytes were examined under a fluorescence microscope.

3. 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 mouse was performed by using either collagenase treatment or a mechanical method. The isolation procedure yielded primary, early secondary and late secondary follicles (Fig. 1). Primary follicles (Fig. 1A; containing only one granulosa cell layer), early secondary follicles (Fig. 1B; two to four granulosa cell layers) and late secondary follicles (Fig. 1C; more than four granulosa cell layers, no antrum) were collected. The effect of different methods of isolating preantral follicles is presented in Fig. 2. As shown in Fig. 2, the 2 ovarian dissociation techniques (mechanical and enzymatic) isolated preantral follicles at different stages of development (primary, early secondary and late se-

condary follicle). The mean recovery number of 75~99 μm isolated by enzymatic method was significantly higher than mechanical method (36.5 and 21.0, respectively). Both 100~124 μm and 125~150 μm of preantral follicle classes obtained after mechanical isolation were significantly higher retrieval number than enzymatic isolation method ($p<0.05$).

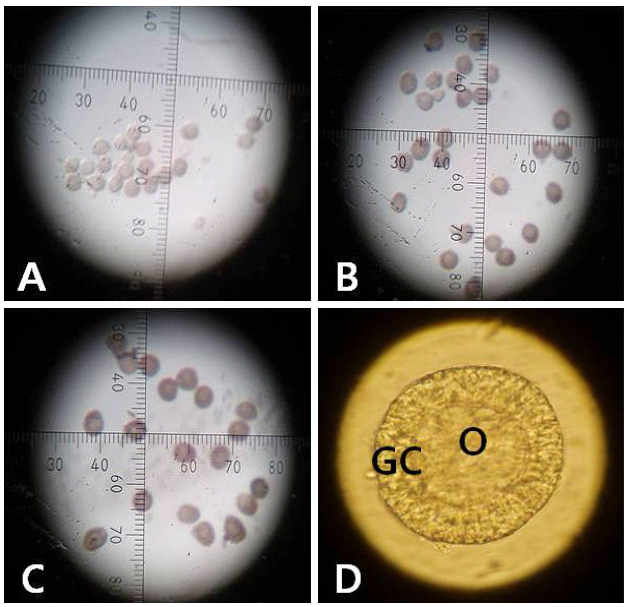

Fig. 1. Morphological characteristics of isolated follicles. (A) Primary follicles consist of an oocyte surrounded by a single layer of cuboidalgranulosa cells. (B) Early secondary follicles are identified by two to four layers of cuboidal granulosa cells around the oocyte. (C) Late secondary follicles are identified by having more than four layers of cuboidal granulosa cells. (D) The follicle consists of a single oocyte (O) and granulosa cells (GC) without antrum.

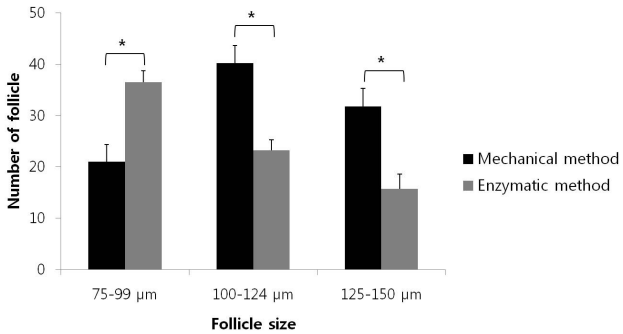

Fig. 2. A comparison of the efficiency of different methods for the collection of mouse follicles at different stages of follicular development. 75~99 μm : primary follicle, 100~124 μm : early secondary follicle, 125~150 μm : late secondary follicle.

Isolated preantral follicles were cultured in the control growth medium for 10 days (Fig. 3). By Day 10, almost all follicles had become attached to the dish and the follicles reached a spread appearance (Fig. 3A, B, C, D and E). After maturation culture of the oocytes grown *in vitro* for 10 days, some of

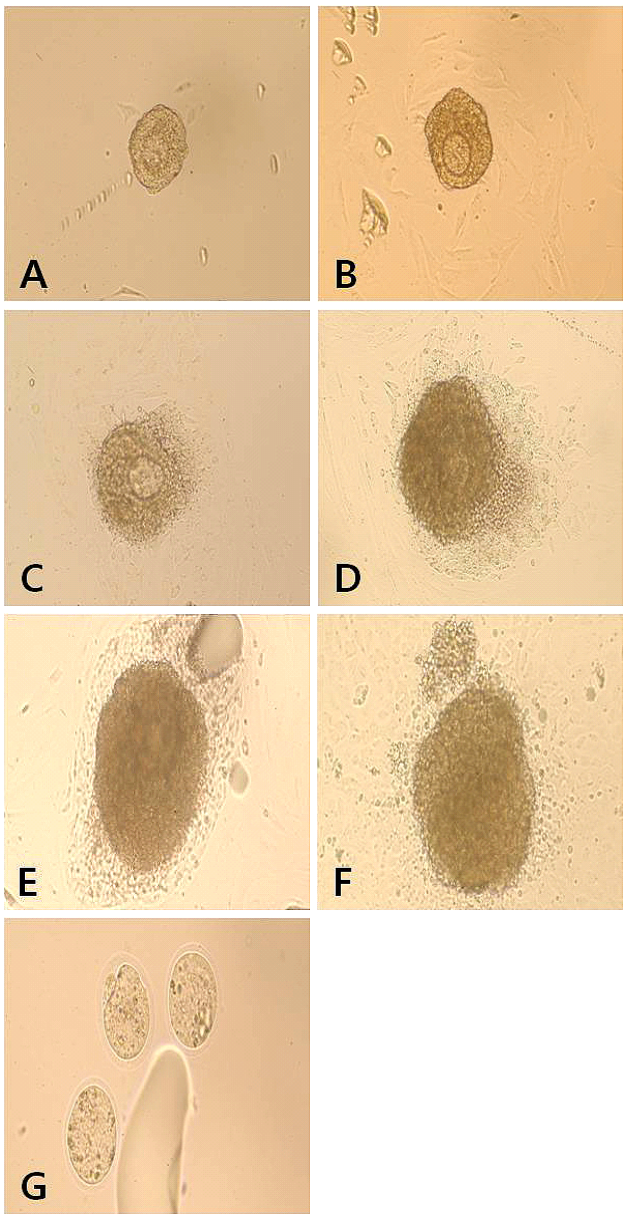

Fig. 3. Morphology of *in vitro* growth and maturation of mouse preantral follicles. A: Preantral follicles cultured for 1 day. B: Preantral follicles cultured for 3 days. C: Day 5 of *in vitro* culture. D: Day 7 of *in vitro* culture. E: Day 9 of *in vitro* culture. F: *In vitro* maturation of a 10 day-culture period ofpreantral follicle. G: After maturation cultured 16~18 h, mature oocyte with extruded polar body.

them matured to metaphase Ⅱ with the first polar body (Fig. 3G). As shown in Table 1, the survival rates of the preantral follicles following *in vitro* growth and maturation demonstrated no difference among the 2 isolation method groups. However, the metaphase Ⅱ rates of the oocytes were significantly higher in mechanical method (17.8%) than in enzymatic method (5.1%).

DISCUSSION

The preantral follicles were isolated for culture from the ovaries of 12-day-old mice. In these ovaries, most of the follicles consist of approximately 1～4 layers of granulosa cells around the oocyte and such oocytes are, at the time of isolation from preantral follicles, in about mid-growth phase and incompetent of undergoing a germinal vesicle breakdown (GVBD) without further development (Eppig and Downs, 1989). Because oocytes in this stage are incompetent to resume meiosis, more time is required for a follicle to develop to the preovulatory stage.

The development of techniques for the isolation of ovarian follicles is vital importance to obtain a large yield of follicles for further *in vitro* culture studies (Telfer, 1996). In this study, we have evaluated a number of possible methods for the isolation of mouse preantral follicles both by mechanical method and by enzymatic digestion. Enzymatic methods are more expensive to perform and isolate large numbers of small follicles (Hartshorne, 1997). Also longer digestion times resulted in a fewer number of preantral follicles. Mechanical method has the disadvantage that it is a very time-consuming procedure and the number of intact follicles per ovary isolated by the physical method was lower (Nayudu and Osborn, 1992; Spears *et al*., 1994). These correspond to our demonstrated in an isolation data on mouse preantral follicles. The recovery of the large size preantral follicles failed in the present study during enzymatic method, which may be due to the location of preantral follicles at different development stages in mouse ovary. A mechanical method was beneficial for the isolation of large amounts of secondary follicles and enzymatic method was beneficial for the isolation of primary follicles.

Another experiment, we have evaluated subsequent follicular development during *in vitro* culture both by mechanical method and enzymatic digestion. Any residual collagenase may affect the ability of isolate follicles to grow and survive *in vitro* (Figueiredo *et al*., 1993). According to Eppig and O'Vrien (1996), a collagenase treatment lost basement membrane and theca cells during isolation procedure. Those two factors may be the reason for the development result of the preantral follicles in this study. Also longer digestion times resulted in a fewer number of preantral follicles (Lim *et al*., 2010). *In vivo*, the basement membrane plays an essential role in maintaining the spherical follicular structure, and its surface increases enormously during follicular growth (30,000 fold in mice) (Gosden *et al*., 1993). Organization of theca cells around the follicle provides structural integrity and helps to establish mesenchymal-epithelial cell interactions between theca cells and granulosa cells (Skinner, 1990). Moreover, maintenance of bidirectional communication between the oocyte and its surrounding somatic cells is vital to the production of a healthy oocyte (Albertini *et al*., 2001; Eppig, 2001). Whereas the fresh follicles isolated mechanically had granulosa cell layers circumscribed by basal lamina and flattened theca cells. And the basement membrane and stromal/thecal tissue are retained in mechanically isolated follicles whereas the enzymatic procedures (such as collagenase) may disrupt the basement membrane (Lucci*et al*., 2002). Since mechanical method may not destroy preantral follicles, that support the growth and differentiation of rodent preantral follicles.

In summary, the techniques for the isolation efficiency of

Table 1. Effect of isolation method on the *in vitro* growth and maturation of mouse preantral follicles

Procedure	No. of follicles cultured	No. (%) of oocytes survived *	GV (%)	GVBD (%)	Meta Ⅱ (%)
Mechanical isolation	101	60 (59.4)	9 (8.9)[a]	33 (32.7)	18 (17.8)[a]
Enzymatic isolation	98	59 (60.2)	23 (23.5)[b]	31 (31.6)	5 (5.1)[b]

GV : Germianl vesicle, GVBD : Germinal vesicle breakdown, Meta Ⅱ : Metaphase Ⅱ.

* Survival was defined as those oocytes (GV, GVBD and metaphase Ⅱ) which showed normal morphology after *in vitro* growth (10 days) and maturation (16～18 h), and was expressed as a percentage of preantral follicles put into culture.

Different lower case letters in the same row (a,b) indicate a significant difference ($p<0.05$).

mouse preantral follicles were examined. Mechanical method, without the use of enzymatic digestion, yielded morphologically normal intact large preantral follicles which were a suitable starting material for culture. More research is necessary to reach the final goal of establishing a culture system that will shorten culture period on follicle growth and produce large quantities of meiotically competent oocytes from preantral follicle.

REFERENCES

Albertini DF, Combelles CM, Benecchi E and Carabatsos MJ. 2001. Cellular basis for paracrine regulation of ovarian follicle development. Reproduction 121:647-653.

Betteridge KJ, Smith C, Stubbings RB, Xu KP and King WA. 1989. Potential genetic improvement of cattle by fertilization of fetal oocytes *in vitro*. J. Reprod. Fertil. Suppl. 38:87-98.

Cortvrindt R, Smitz J and Van Steirteghem AC. 1996. *In-vitro* maturation, fertilization and embryo development of immature oocytes from early preantral follicles from prepuberal mice in a simplified culture system. Hum. Reprod. 11: 2656-2666.

Driancourt MA. 1991. Follicular dynamics in sheep and cattle. Theriogenology 35:55-79.

Eppig JJ and Downs SM. 1987. The effect of hypoxanthine on mouse oocyte growth and development *in vitro*: maintenance of meiotic arrest and gonadotrophin-induced oocyte maturation. Dev. Biol. 119:313-321.

Eppig JJ and O'Brien MJ. 1996. Development *in vitro* of mouse oocytes from primordial follicles. Biol. Reprod. 54:197-207.

Eppig JJ and Schroeder AC. 1989. Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation, and fertilization *in vitro*. Biol. Reprod. 41:268-276.

Eppig JJ. 2001. Oocyte control of ovarian follicular development and function in mammals. Reproduction 122:829-838.

Figueiredo JR, Hulshof SCJ, Hurk R. van den, Ectors FJ, Fontes RS, Nusgens B, Bevers MM and Beckers JF. 1993. Development of a combined new mechanical and enzymatic method for the isolation of intact preantral follicles from tetal, calf and adult bovine ovaries. Theriogenology 40: 789-799.

Gong SP and Lim JM. 2009. Effects of [beta]-mercaptoethanol on the growth of preantral follicles and the maturation of intrafollicular oocytes. Asian-Aust. J. Anim. Sci. 22:35-41.

Gosden RG, Boland NI, Spears N, Murray AA, Chapman M, Wade JC, Zohdy NI and Brown N. 1993.The biology and technology of follicular oocyte development *in vitro*. Reprod. Med. Rev. 2:129-152.

Gougeon A. 2003. The early stages of follicular growth. In: Trounson AO, Gosen RG, editors. Biology and Pathology of the Oocyte. Cambridge University Press, pp 29-43.

Hartshorne GM. 1997. *In vitro* culture of ovarian follicles. J. Reprod. Ferti. 2:94-104.

Johnston LA, Donoghue AM, O'Brien SJ and Wildt DE. 1991. Rescue and maturation *in vitro* of follicular oocytes collected from nondomestic felid species. Biol. Reprod. 45:898-906.

Ksiazkiewicz LK. 2006. Recent accievements in *in vitro* culture and preservation of ovarian follicles in mammals. Biol. Reprod. 6:3-16.

Lim HJ, Kim DH, Im GS and Lim JM. 2010. Comparison of mechanical and enzymatic methods for the isolation of bovine ovarian follicles. J. Emb. Trans. 25:93-96.

Lucci CM, Rumpf R, Figueiredo FR and Bao SN. 2002. Zebu (Bosindicus) ovarian preantral follicles: Morphological characterization and development of an efficient isolation method. Theriogenology 57:1467-1483.

Mariana JC and Nguyen HN. 1973. Folliculogenese chez la vache. Ann. Biol. Anim. Bioch. Biophys. 13:211-221.

Marion GB, Gier HB and Choudary JB. 1968. Micromorphology of the bovine ovarian follicular system. J. Anim. Sciences. 27:451-465.

Nayudu PL and Osborn SM. 1992. Factors influencing the rate of preantral and antral growth of mouse ovarian follicles *in vitro*. J. Reprod. Fertil. 95:349-362.

Saumande J. 1981. Ovogeneseet folliculogenese. Rec. Med. Vet. 157:29-38.

Skinner MK. 1990. Mesenchymal (stromal)-epithelial cell interactions in the testis and ovary which regulate gonadal function. Reprod. Fertil. Dev. 2:237-243.

Spears N, Boland NIMurray AA and Gosden RG. 1994. Mouse oocytes derived from *in vitro* growth primary ovarian follicles are fertile. Hum. Reprod. 9:527-532.

Telfer EE. 1996. The development of methods for isolation and culture of preantral follicles from bovine and porcine ovaries. Theriogenology 45:101-110.

Webber LJ, Stubbs S, Stark J, Trew GH, Margar R, Hardy K and Franks S. 2003. Formation and early development of follicles in the polycystic ovary. Lancet. 362:1017-1021.

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