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In Vitro Maturation, Fertilization and Development of Mouse Oocytes Derived from *In Vitro* Grown Preantral Follicles

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체외성장된 Preantral Follicle에서 유래된 생쥐난자의 체외성숙, 수정 및 발달

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ABSTRACT

The objective of this study was to examine maturation, fertilization and developmental rate of the *in vitro*-grown mouse oocytes, and to compare these results with those of oocytes grown and matured *in vivo*. The preantral follicles isolated from 12-day-old mice were cultured on Transwell-COL membrane inserts. After *in vitro* growth and maturation, 72.5 % of oocytes grown *in vitro* produced polar body which can be comparable to *in vivo* growth (70.5 %). However, the mean oocyte diameter of the *in vitro* group (69.6±2.1 μm) was smaller than that of the *in vivo* group (73.3±3.0 μm). The fertilization rate was significantly lower ($p<0.05$) in the *in vitro* group (76.5%) than in the *in vivo* group (90.2%), however, there was no difference in the percentage of monospermic and polyspermic oocytes between two groups. The capacities of *in vitro* grown ova to cleave and develop to blastocyst were (57.8 and 14.4%, respectively) significantly lower ($p<0.001$) than those of the *in vivo* counterpart (84.4 and 56.6%, respectively). Moreover, the mean number of cells per blastocyst was significantly lower ($p<0.05$) in the *in vitro* group (39.0±10.8) than in the *in vivo* group (60.5±12.5). Live young were produced from transferred 2-cell embryos derived from *in vitro*-grown and matured oocytes. In conclusion, the results show that *in vitro*-grown oocytes did not achieve the developmental capacity of *in vivo*-grown oocytes.

(Key words: Mouse, *In vitro* growth, Preantral follicle, Live young)

Ⅰ. INTRODUCTION

There are thousands of oocytes present in the ovaries of mammals. However, most of them undergo degeneration during the growth and maturation, only a very few oocytes become available for ovulation (Gosden and Telfer, 1987). *In vitro* culture of preantral follicles isolated from ovaries has been thought to be a potentially useful stratagie to escape

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the natural process of follicular atresia (Eppig et al., 1990). The mammalian oocytes isolated from antral follicles can mature, be fertilized and developed into live young mice (Schroeder and Eppig, 1984), rats (Vanderhyden and Armstrong, 1989), sheep (Staigmiller and Moor, 1984), cattle (Goto et al., 1988), pigs (Mattioli et al., 1989) and human (Cha et al., 1991). However, the mammalian ovaries have only limited number of antral follicles available for the *in vitro* maturation.

The ovarian preantral follicles are an enormous and potentially valuable source of oocytes that could be used for clinical, agricultural and zoological purposes. However, developing optimal *in vitro* culture technique to bring the immature oocytes in preantral follicles to mature oocytes is an important prerequisite. To date, many researchers published their efforts to develop culture technique and their insights into the metabolism of follicles, production of steroids during the growth, and effects of gonadotrophins and growth factors on follicular development of mouse (Torrance et al., 1989; Carroll et al., 1991, Nayudu and Osborn, 1992; Boland et al., 1993; Boland and Gosden, 1994; Hartshorne et al., 1994; Cortvrindt et al., 1996), cattle (Figueiredo et al., 1993), pig (Hirao et al., 1994) and even human (Roy and Treacy, 1993).

Murine species have been widely used to develop the culture system for preantral follicles and to study growth of follicle and oocyte, since they are small, readily available and grow to Graafian follicle within a short time-span. Particularly, the studies for the culture of mouse preantral follicles have demonstrated that not only the growth and maturation of the oocyte but also fertilization and development were possible (Eppig and Schroeder, 1989; Spears et al., 1994: Cortvrindt et al., 1996). Furthermore, live young were obtained by the transfer of embryos derived from *in vitro* grown oocytes (Eppig and Schroeder, 1989; Spears et al., 1994), although these studies demonstrated that mouse oocytes grown, matured and fertilized *in vitro* have limited developmental capacity.

The aim of this study was to examine maturation, fertilization and developmental rates of the mouse oocytes derived from the *in vitro* grown preantral follicles, and to compared these findings with those obtained from oocytes grown and matured *in vivo*.

Ⅱ. MATERIALS AND METHODS

1. Isolation of Preantral Follicles

The ovaries were aseptically removed from 12-day-old ICR female mice. They were immersed into Leibovitz L-15 medium containing 1 mg/ml collagenase (C-2674, Sigma) and 0.2 mg/ml DNase I (DN-25, Sigma) for 20 min at 37℃ and repeatedly drawn in and out of the pipette until the ovaries were dissociated into individual follicles (Fig. 1A) (Eppig and O'Brien, 1996). The follicles to be cultured were selected by the following criteria: i) intact round follicular structure with two to three layers of granulosa cells; ii) the oocyte had to be visible, round and centrally located within the follicle.

2. *In Vitro* Growth and Maturation of Preantral Follicles

The culture medium was MEM medium supplemented with 5% fetal bovine serum, 100 mIU/ml FSH (Metrodin-HP, Sereno) and 10 mIU/ml LH (L-5259, Sigma) (Kim et al., 1999). The follicles were cultured on Transwell-COL membrane inserts (3.0 μm pore size, 24.5 mm diameter; Costar) in six well cluster dishes to prevent the loss of structural integrity between the oocyte and granulosa cells (Eppig and Schroeder, 1989). The follicles were cultured for 10 days at 37℃ and 5% CO_2 in air (Fig. 1B). Half of the medium was changed every 2 days. After 10 days of growth *in vitro*, follicles

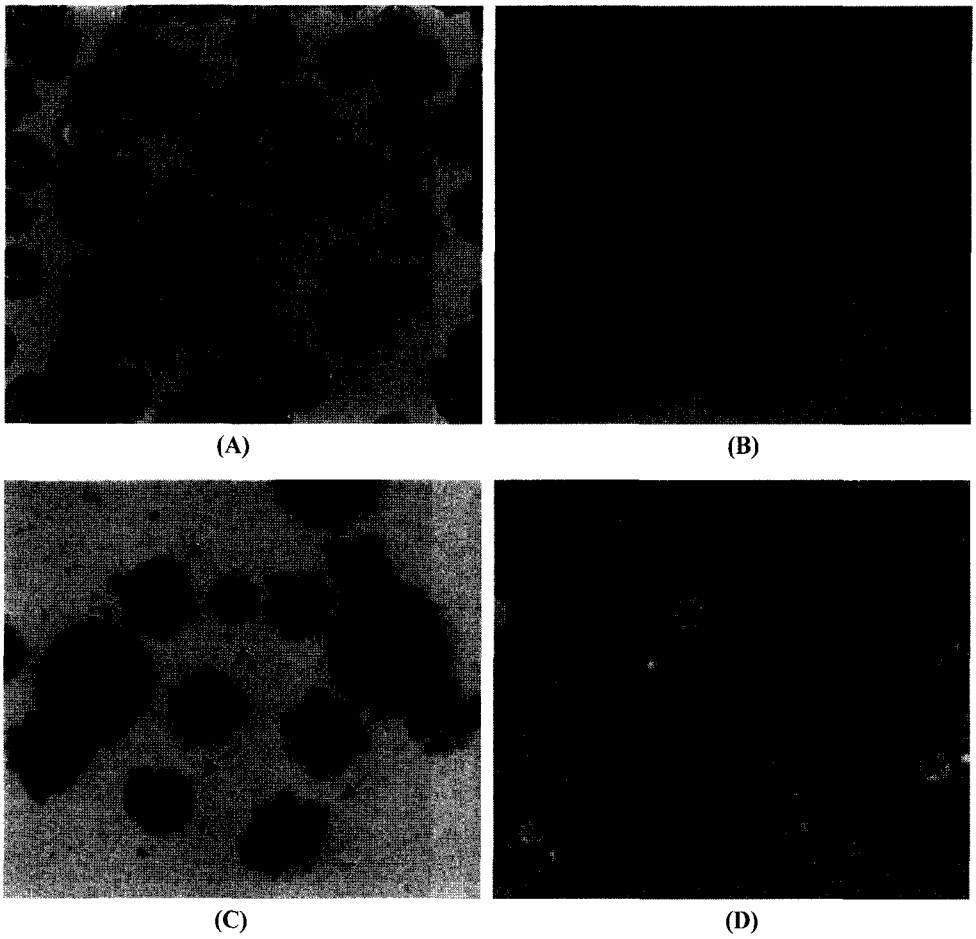

Fig. 1. ***In vitro*** **growth and maturation of mouse preantral follicles. (A) Preantral follicles isolated from 12-day-old mice (×100). (B) Preantral follicles after 10 days of culture (×100). (C)** ***In vitro*** **maturation of the preantral follicles cultured for 10 days: mucified oocyte-cumulus complex floating free in the culture medium (arrow) (×100). (D) Mature oocytes derived from the** ***in vitro*****-grown preantral follicles (×400).**

were allowed to mature for 16~18 hours in medium supplemented with 1.5 IU/ml hCG (Profasi, Sereno) (Fig. 1C and D).

3. *In Vitro* Fertilization

In vitro-matured oocyte-granulosa cell complexes were placed into 50 μl drops of Whittingham's T_6 medium supplemented with 30 mg/ml bovine serum albumin (A-3311, Sigma) under mineral oil.

As a control, *in vivo*-matured oocytes were obtained from superovulated 23-day-old ICR mice.

The sperms collected from the cauda epididymis of mature ICR male mice were incubated for 2 hr in fertilization medium before insemination. The oocytes were inseminated with spermatozoa with concentration of $1\sim2\times10^6$/ml. Four hours after the insemination, oocytes were washed and cultured for *in vitro* development.

Six hours after insemination, a part of oocytes were fixed in 2% formaldehyde in PBS and stained

with 2.5 μg/ml Hoechst 33342. The detailed status of the oocytes was evaluated under a fluorescence microscope.

4. *In Vitro* Development

The fertilized oocytes were cultured in 20 μl drops of KSOM medium supplemented with 10% (v/v) serum substitute supplement (SSS, Irvine), 1% (v/v) essential amino acids and 0.5% (v/v) non -essential amino acids under mineral oil. The oocytes were examined the following day, and the number of 2-cell embryos was noted. The 2-cell embryos were further cultured for 4 days until the blastocyst stage.

The blastocysts were fixed in 2% formaldehyde in PBS and stained with 2.5 μg/ml Hoechst 33342 to count the number of cell. The cell number of blastocyst was examined under a fluorescence microscope.

5. Embryo Transfer

Morphologically normal 2-cell embryos that derived from *in vitro* growth, maturation and fertilization were transferred to the oviduct of pseudopregnant female ICR recipients.

6. Statistical Analysis

The statistical significance of the data was analyzed using Students t-test and the chi-square (χ^2) test. Statistical significance was established at the $P<.05$ level.

Ⅲ. RESULTS

1. *In Vitro* Growth and Maturation of Oocytes within Preantral Follicles

The percentage of the oocytes that had undergone germinal vesicle breakdown (GVBD) and had extruded the first polar body (Metaphase Ⅱ) was determined. As shown in Table 1, the percentage of metaphase Ⅱ oocytes of the *in vitro* group (72.5%) was not difference from that of the *in vivo* group (70.5%). The oocytes isolated from 12-day-old mice were 56.7±2.5 μm (mean±SEM) in diameter, and the mean size had increased during the culture period (Fig. 1). However, the mature oocyte of the *in vitro* group (69.6±2.1 μm) were smaller than the mature oocyte of equivalent chronological age *in vivo* group (23 days old; 73.3±3.0 μm).

2. *In Vitro* Fertilization of the *In Vitro*-Grown and Matured Oocytes

As shown in Table 2, the fertilization rates of *in vitro* group (76.5%) was significantly lower ($P<0.05$) than that of *in vivo* group (90.2%). The percentage of normal monospermic oocytes in the fertilized oocytes was not difference between the two groups (84.6 vs 85.5%). The percentage of polyspermic oocytes was also similar between the two groups (15.4 vs 14.5%).

Table 1. Maturation rates of oocytes derived from *in vitro*- and *in vivo*-grown mouse preantral follicles

Source	No. of follicles cultured	No.(%) of oocytes survived	GV(%)[1]	GVBD(%)[2]	Meta Ⅱ(%)[3]	Oocyte diameter(μm)
In vitro	147	109 (74.1)[a]	20 (18.3)	10 (9.2)	79 (72.5)	69.6±2.1
*In vivo**	105	105 (100)[b]	8 (17.1)	13 (12.4)	74 (70.5)	73.3±3.0

[a,b] $P<0.05$

[1]: Germinal Vesicle, [2]: Germinal Vesicle Break-Down, [3]: Metaphase Ⅱ

* Oocytes were collected from the ovaries of 22-day-old mice and matured in medium containing 1.5 IU/ml hCG for 16~18 hr.

Table 2. Fertilization rates of mouse oocytes grown and matured *in vitro* and *in vivo*

Source	No. of oocytes examined	No. (%) of oocytes fertilized		
		Total	Monospermic	Polyspermic
In vitro	85	65 (76.5)[a]	55 (84.6)	10 (15.4)
In vivo	92	83 (90.2)[b]	71 (85.5)	12 (14.5)

[a,b] $P<0.05$.

3. *In Vitro* Development of the *In Vitro*-Grown, Matured and Fertilized Oocytes

The embryos cleaved to the 2-cell stage after insemination, as shown in Table 3, *in vitro* group (57.8%) was significantly lower ($P<0.001$) than those of *in vivo* group (84.4%). When the embryos were examined 5 days after insemination, 14.4% of the *in vitro*-grown oocytes developed to the blastocyst stage. It was significantly less ($P<0.001$) than that of the *in vivo*-grown oocytes (56.6%). As shown in Table 4, total cell number of the blastocysts derived from the *in vitro*-grown oocytes (39.0±10.8) were significantly lower ($P<0.05$) than those of blastocysts derived from the *in vivo*-grown oocytes (60.5±12.5).

Table 4. Number of cells per mouse blastocyst derived from *in vitro*- and *in vivo*-grown oocytes

Source	No. of blastocysts examined	No. of cells (mean ± SEM)	Range
In vitro	35	39.0±10.8[a]	21～61
In vivo	32	60.5±12.5[b]	42～85

[a,b] $P<0.05$.

4. Embryo Transfers

The result of embryo transfer is shown in Table 5. After fertilization of oocytes derived from the *in vitro* growth and maturation, 181 2-cell embryos were transferred to the oviducts of 8 pseudopregnant recipients (15～38 embryos per recipient), and 2 recipients became pregnant. Six live pups (3.3%) were born, and they were three males and three females. These pups appeared to be normal and proved to be fertile after puberty.

Table 3. Developmental rates of mouse oocytes grown and matured *in vitro* and *in vivo* after *in vitro* fertilization

Source	No. of oocytes examined	No. (%) of embryos developed to			
		2-cell	4-cell	Morular	Blastocyst
In vitro	232	134 (57.8)[a]	96 (41.4)[a]	62 (26.7)[a]	33 (14.2)[a]
In vivo	205	173 (84.4)[b]	162 (79.0)[b]	133 (64.9)[b]	116 (56.6)[b]

[a,b] $P<0.001$.

Table 5. Result of embryo transfer of the *in vitro* growth, maturation and fertilization derived-embryos

No. of recipients	No. of embryos transferred	No. of pregnant	No.(%) of offsprings	
8	181	2	6 (3.3)	Male: 3, Female: 3

Ⅳ. DISCUSSION

Preantral follicles for culture were isolated from ovaries of 12-day-old mice. In these ovaries, most of the follicles consist of about 1~3 layer of granulosa cells around the oocyte. Such oocytes are in about mid-growth phase and are incompetent to undergo germinal vesicle breakdown (GVBD) at the time of isolation (Eppig and Down, 1987), however follicular cells possess all the morphological and functional ability for sustaining follicles growth. In this experiment, we also confirmed that oocytes were, at the time of isolation from preantral follicles, not capable of resuming meiosis.

According to the experimental purpose, various culture methods of growing preantral follicles to maturity *in vitro* have been developed, such as agar or collagen gel embedding (Torrance et al., 1989; Carroll et al., 1991; Roy and Treacy, 1993), collagen impregnated membranes (Eppig and Schroeder, 1989), agar coated plastic petri dishes (Hirao et al., 1990), 96-V-well microtitre plates (Spears et al., 1994; Hartshorne et al., 1994) and micro droplets covered with mineral oil (Cortvrindt et al., 1996). In this experiment, we used collagen coated Transwell-COL membrane inserts (Eppig and Schroeder, 1989) for culture of mouse preantral follicles. These membranes were treated with an equimolar mixture of Type I and Ⅲ collagen produced from bovine placentae, and allowed attachment and maintenance of the complexes with only minimal migration of the granulosa cells from the oocytes compared to general petri dishes. Generally, preantral follicles isolated by enzyme treatment is more difficult to maintain three-dimensional structure between the oocyte and granulosa cells because theca cells and basal lamina were partially damaged by collagenase. However, culture of preantral follicles on Transwell-COL membrane makes it possible for oocyte and granulosa cells to maintain three-dimensional structure as a complex, which helps survival and growth of oocytes and follicles.

FSH is one of the most important factors for the growth of follicles in ovaries. It is known that addition of FSH in culture medium plays an important role in survival and maturation of follicles and oocytes and also alleviates oxidative stress by enhancing superoxide dismutase and other scavenger systems (Tilly and Tilly, 1995). Previous studies on the culture of mouse preantral follicles have reported that FSH promotes the growth of follicle and the formation of antrum (Hartshorne et al., 1994) by stimulating proliferation of granulosa cells, increases GVBD and extrusion of the first polar body of the *in vitro* grown oocytes (Eppig and Schroeder, 1989) and significantly increases the production of estrogen and lactate of follicles (Nayudu and Osborn, 1992; Boland et al., 1993). Our experiment confirmed that FSH plays key role in survival and growth of mouse preantral follicles. It was observed that, without FSH, the three-dimensional structure between oocyte and granulosa cells collapses and then follicles degenerates due to poor proliferation of granulosa cells (Kim et al., 1999). LH is also an important factor for the growth of granulosa cells (Yong et al., 1992), the formation of antrum and the production of estrogen (Qvist et al., 1990). Addition of LH to FSH throughout the culture period increaes antral-like cavity formation of follicles and maturation rates of the *in vitro*-grown oocytes (Cortvrindt et al., 1998). Besides, it is known that proper LH:FSH ratio, most likely 1:10, has a positive effect on survival of follicles and maturation of oocytes (Kim et al., 1999). Based upon this findings, we added 10 mIU/ml LH and 100 mIU/ml FSH to obtain higher survival rates of follicles and higher maturation rate of oocytes and found that maturation rate of the *in vitro*-grown oocytes is similar to that of the *in vivo*-grown

oocytes. It was confirmed that gonadotrophins are essential factors on the survival and growth of preantral follicles.

It is reported that oocytes grown and matured *in vitro* have reduced capacity to be fertilized (Spears et al., 1994) and higher rate of abnormal fertilization (Hirao et al., 1990) as compare with their *in vivo* counterparts. In this experiment, we confirmed that oocytes grown and matured *in vitro* has lower rate of fertilization than oocytes grown and matured *in vivo*. This phenomenon is probably due to incomplete cytoplasmic maturation of oocytes (Hirao et al., 1990) and the failure of development of the activation mechanism triggered by sperm penetration. It is assumed that long-term *in vitro* culture results hardening of the zona pellucida, thereby prevent the penetration of the sperm into the oocyte. This assumption is supported by the observation that the time required for digestion of zona pellucida of oocytes grown and matured *in vitro*, using α -chymotrypsin, was longer than *in vivo* grown oocytes (unpublished data).

According to the previous studies about developmental capacity of oocytes grown and matured *in vitro*, less than 50% of fertilized oocytes underwent development to the blastocyst stage (Eppig and Schroeder, 1989, Spears et al., 1994). In this experiment, the rates of development to blastocysts is only 12%. The poorer rate of blastocyst development could be explained by three factors. The first factor is the difference of mouse strain used. We used ICR strain for experiment while many other researchers had used F_1 hybrid strain. The second is the difference of O_2 concentration in culture condition. It is known that concentration of 20% O_2 is an optimal concentration for the culture of intact preantral follicles isolated by manual dissection (Smitz et al., 1996). On the contrary, for preantral follicles isolated by enzyme treatment, 5% O_2 is believed as an optimal concentration because partial damage of theca cells and basal lamina cause more oxidative stress (Eppig and Wigglesworth, 1995). Finally, we did not add EGF in maturation medium. Previous studies for the culture of preantral follicles showed impressive beneficial effects of EGF, combined with FSH or LH, on maturation, fertilization and development to blastocysts (Boland and Gosden, 1994; Spears et al., 1994; Smitz et al., 1998; De La Fuente et al., 1999).

There had been several reports of production of live young after transfer of embryos derived from the *in vitro*-grown, matured and fertilized oocytes. However, about 5 % of the transferred embryos developed to the live young (Eppig and Schroeder, 1989, Spears et al., 1994). We also could produce live young from oocytes grown *in vitro* from preantral follicles by transfer of 2-cell embryos, and the rates of live young production was 3.3%. It is considered that lower live young production rate after *in vitro* maturation is related with smaller cell number of the blastocyst compared to *in vivo* counterparts. The total cell number is regarded as an important index for developmental capacity of blastocyst. Meanwhile, this first report that oocytes from preantral follicles of ICR strain mouse can growth and mature, be fertilized and develop into live young.

The present study confirmed that oocytes from preantral follicles of mouse can complete growth and maturation *in vitro* and live young can be produced after fertilization *in vitro* and transfer to recipient. However, the developmental capacity of the *in vitro* grown oocyte was not comparable to that of *in vivo*-grown oocytes. This result provided an explanation why only a few *in vitro*-grown oocytes could develop into live young. Further studies is needed to improve the culture system for preantral follicles and verify the cause of the lower developmental capacity of *in vitro*-grown oocytes.

Ⅴ. 요 약

본 연구의 목적은 체외성장된 생쥐 preantral follicle 내에 존재하는 난자의 성숙율, 수정율, 배발달율을 조사하는 것이었으며, 그리고 이러한 결과들을 체내 성장된 난자와 비교하는 것이었다. Preantral follicle은 생후 12일령된 생쥐로부터 분리하였으며, 분리된 preantral follicle은 Transwell -COL membrane insert에서 배양을 실시하였다. 체외성장 및 성숙 후, 제 1극체를 방출한 metaphase Ⅱ 난자는 72.5%로서 체내성장된 난자의 70.5%에 비하여 차이가 없는 것으로 나타났다. 그러나 난자 직경은 체외성장군 (69.6±2.1 μm)이 체외성장군 (73.3 ± 3.0 μm)에 비하여 작은 것으로 나타났다. 체외수정율은 체외성장군 (76.5%)이 체내성장군 (90.2%)에 비하여 유의하게 낮았지만, 다정자 수정된 난자의 비율은 두 군간에 차이가 없었다. 배반포까지의 배발달율은 체외성장군 (14.4%)이 체내성장군 (56.6%)에 비하여 유의하게 낮았으며, 또한 배반포의 세포수에 있어서도 체외성장군 (39.0±10.8)이 체내성장군 (60.5±12.5)에 비하여 유의하게 작은 것으로 나타났다. 체외성장 및 성숙 유래의 2-세포기 수정란을 이식한 결과, 산자 생산을 확인할 수 있었다. 결론적으로 이러한 결과는 체외성장된 난자는 체내성장된 난자와 같은 발생능력을 갖지 못함을 보여주고 있다.

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