FULL PAPER *Theriogenology*

In Vitro Growth of Mouse Ovarian Preantral Follicles and the Capacity of Their Oocytes to Develop to the Blastocyst Stage

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ABSTRACT. Two groups of mouse preantral follicles with diameters of 125–150 and 151–175 μm were cultured individually for 6 days in a medium supplemented with FSH and fetal calf serum to determine their *in vitro* growth characteristics. Their oocyte capacity for maturation and development to the blastocyst stage following *in vitro* fertilization was also assessed. Antral formation rate at the end of culture was higher in the follicles of 151–175 μm (89%) than 125–150 μm (76%). The timing of antrum formation was different between the two follicle categories: most 151–175 μm follicles formed antra earlier than 125–150 μm follicles (days 4 and 5 vs. 5 and 6). However, follicle diameters at the time of antrum formation were the same regardless of the initial size and the culture period. Maturation rates of the oocytes derived from both categories of *in vitro* grown follicles (70 and 62%) were not different from those of oocytes from *in vivo* grown follicles (74%). The *in vitro* derived oocytes, however, showed less cleavage (30 and 35%) than the *in vivo* derived oocytes (89%). Although the oocytes from both follicle categories developed to the morula stage after *in vitro* fertilization, blastocysts were only obtained from oocytes derived from the 151–175 μm category. These results demonstrate that an individual follicle culture system using a medium with FSH and fetal calf serum supports *in vitro* growth of mouse preantral follicles with diameters of 151–175 μm to the preovulatory stage, and that their oocytes have the capability to develop to the blastocyst stage.

KEY WORDS: culture, development, growth, mouse, preantral follicle.

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In vitro culture systems for mouse preantral follicles have utilized group, individual, spherical or non-spherical culture methods [12]. The collective culture of oocyte-granulosa cell complexes from prepubertal mice has potential for oocyte farming, since abundant fertile oocytes can be produced from preantral follicles by this method [5]. The first live offspring derived from oocytes of *in vitro* grown follicles were obtained with this culture system [6]. The individual culture of mechanically isolated preantral follicles maintains an intact and spherical follicular structure during a 5- to 6-day period [3]. This method of follicle culture has advantages for investigating mechanisms of folliculogenesis, oocyte growth, and ovarian dysfunctions over group and non-spherical culture. The growth patterns of follicles in this culture system were reported to be similar to those observed *in vivo* [16] or faster [13] when follicles were cultured in a medium supplemented with mouse serum and follicle stimulating hormone (FSH). High *in vitro* ovulation rates [19] and live offspring [20] have also been obtained by using this method.

Most previous studies using the individual/spherical follicle culture system focused on follicles with an initial diameter of 170 to 250 μm [3, 13–16, 19–21]. The population of smaller follicles (<160 μm) in adult mouse ovaries is bigger than that of larger follicles (>160 μm) [10]. However, there is limited information on the capacity for maturation, fertilization, and subsequent development of oocytes derived from small preantral follicles (<160 μm) in the individual/spherical follicle culture system. The present study focused on the smaller (125–175 μm) mouse preantral follicles and evaluated their growth using a culture system which maintains an intact and spherical follicle structure *in vitro*. The capacity of the oocytes for meiotic resumption and subsequent embryonic development was also assessed.

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MATERIALS AND METHODS

Animals: Adult C57BL/6J female and CBA male mice purchased from Japan SLC Inc. (Shizuoka, Japan) were bred to obtain F1 mice. Four- to six-week-old F1 females were used to collect preantral and antral follicles. Eight- to nine-week-old F1 males were used to collect epididymal spermatozoa. These mice were housed in a temperature- and light-controlled room at 23°C with a 14 hr light: 10 hr dark photoperiod and provided with feed and water *ad libitum*. The handling of animals was done according to the guidelines laid down by the university.

Preantral follicle isolation: Mice were killed by cervical dislocation. Ovaries were removed to Leibovitz L-15 medium (Sigma, St. Louis, MO, U.S.A.) supplemented with 3 mg/m*l* bovine serum albumin (BSA; fraction V, Sigma), 75 μg/m*l* penicillin and 50 μg/m*l* streptomycin in a 60 mm petri dish (Falcon 1007; Becton Dickinson, Franklin Lakes, NJ, U.S.A.) at 37°C. Preantral follicles were isolated by mechanical microdissection using 25G needles attached to 1 m*l* syringes. Follicles with diameters of 125–175 μm without irregularities (*i.e.*, non-spherical or non-centrally located oocytes with dark granulosa layers) were selected. Mean

follicle diameter excluding the theca stroma was estimated by measuring two perpendicular diameters (length and width) at × 200 magnification with a pre-calibrated ocular micrometer under an inverted microscope (TMS, Nikon, Tokyo, Japan). In a separate study, the follicles of 125–150 μm were classified as type 4 and most 151–175 μm follicles as type 5a according to criteria described elsewhere [18]. Therefore, follicles were allocated to one of the two size categories, 125–150 and 151–175 μm, based on diameter (average of the length and width).

Preantral follicle culture: Preantral follicles were cultured for 6 days individually in 25 μl drops of α-minimal essential medium (α-MEM; Gibco, Grand Islands, NY, U.S.A.) supplemented with 5% heat-inactivated fetal calf serum (FCS; Gibco), 0.1 IU/ml porcine follicle stimulating hormone (pFSH; Antrin, Denka Pharmaceutical Co., Ltd., Kanagawa, Japan), 10 ng/ml human recombinant epidermal growth factor (hrEGF; Sigma), 75 μg/ml penicillin and 50 μg/ml streptomycin under paraffin oil in a humidified atmosphere of 5% CO_2 in air at 37°C. Follicles were transferred to fresh drops of culture medium every 24 hr.

Collection of cumulus-oocyte complexes from in vitro and in vivo grown follicles: Cumulus-oocyte complexes (COCs) derived from *in vitro* grown antral follicles were recovered 6 days after the initiation of preantral follicle culture. To collect COCs from *in vivo* grown antral follicles, mice were primed with an intraperitoneal injection of 5 IU equine chorionic gonadotropin (Serotropin, Teikoku Hormone Mfg. Co., Ltd., Tokyo, Japan). Forty-four hours later, the ovaries were removed as described above. COCs were isolated by puncturing the antral follicles grown *in vitro* and *in vivo* using a 25G needle attached to a 1 ml syringe.

In vitro maturation of oocytes: COCs isolated from *in vitro* and *in vivo* grown antral follicles were incubated for 16 hr in 100 μl drops (≤50 COCs/drop) of α-MEM supplemented with 5% FCS, 1 IU/ml pFSH, 20 ng/ml hrEGF, 1 IU/ml human chorionic gonadotropin, 75 μg/ml penicillin and 50 μg/ml streptomycin under paraffin oil at 37°C in a humidified atmosphere of 5% CO_2 in air. The nuclear maturation of the oocytes was assessed after denudation by repeated pipetting. Oocytes were scored as having a germinal vesicle (GV) when the vesicle was recognized, as undergoing GV breakdown when the GV was absent, and as being in metaphase II (M II) when the first polar body was present.

In vitro fertilization and culture of oocytes: A sperm suspension was prepared using the spermatozoa collected from the cauda epididymis of mature F1 males and pre-incubated at a concentration of $3.6–5.0 \times 10^7$ cells/ml for 1 hr in 500 μl of TYH medium [22] supplemented with 3 mg/ml BSA to induce sperm capacitation. The COCs were put into 500 μl of TYH medium for fertilization and co-incubated with pre-incubated spermatozoa ($1–2 \times 10^5$ cells/ml) for 4 hr in a humidified atmosphere of 5% CO_2 in air at 37°C. The oocytes were then washed in KSOM supplemented with 3 mg/ml BSA [8] and cultured in 25 μl droplets of the same medium (5–15 oocytes/drop) at 37°C in an atmosphere of 5% CO_2 in air under paraffin oil for 5 days. The developmental stages of inseminated oocytes were determined by morphological evaluation every 24 hr under an inverted microscope.

Assessment of steroid hormone: The spent medium from follicle cultures was assayed for estradiol-17β, progesterone and testosterone by enzyme immunoassay. Two hundred microliters of sample (150 μl when the follicle number was low) were extracted with 1 ml of diethyl ether and reconstituted with assay buffer. The reconstitutes were diluted 1:2 for the estradiol-17β assay in samples from antral stage follicles. The assay sensitivities were 17.2 pg/well for estradiol-17β, 4.3 pg/well for progesterone and 1.1 pg/well for testosterone. The intra- and inter-assay coefficients of variation were 4.9 and 6.8% for estradiol-17β, 3.9 and 6.5% for progesterone, and 7.1 and 8.9% for testosterone, respectively.

Study 1: *In vitro* growth and steroid hormone production of preantral follicles were determined. In the first experiment, the follicles were examined for the presence of antrum daily to determine the timing of antrum formation in relation to the size of follicles at the start of culture. In the second experiment, *in vitro* growth of follicles was determined daily by measuring the diameter of follicles. Fifteen microliters of spent medium were collected daily from each culture droplet after transferring the follicles to fresh medium to determine the steroid production of cultured follicles. At the time of antrum formation and thereafter, spent medium was pooled from the culture droplets in which follicles had shown antrum formation on the same day of culture. The samples were frozen at –80°C until hormone assay.

Study 2: Maturation and subsequent developmental capacity of oocytes derived from *in vitro* grown follicles were examined. First, COCs recovered from *in vitro* and *in vivo* grown antral follicles were cultured for maturation to examine the oocyte capacity for meiotic resumption. In the next experiment, the oocytes derived from *in vitro* and *in vivo* grown antral follicles were inseminated to assess their fertilizability and subsequent capacity to develop to the blastocyst stage.

Statistical analysis: The antrum formation, maturation and cleavage rates were compared by Fisher's exact test. Follicle diameters and growth rates were compared by one-way analysis of variance followed by Fisher's protected least significant difference as a post hoc test.

RESULTS

Study 1: As shown in Table 1, the antrum formation rate at the end of culture (Day 6) was lower in the follicles of 125–150 μm than in those of 151–175 μm ($p<0.05$). This was due to the higher incidence (14.5%) of premature oocyte extrusion, outgrowth and degeneration in the follicles of 125–150 μm compared to the follicles of 151–175 μm (6.8%). Most antrum formation occurred over a 2-day period in both categories: on days 5 and 6 in 125–150 μm follicles, and on days 4 and 5 in 151–175 μm follicles.

In the next experiment, therefore, we focused on these

Table 1. Frequency and timing of antrum formation in preantral follicles during *in vitro* culture

Initial size (μm)	No. of follicles cultured (replicates)	% of follicles forming antra on each day of culture						Total % of antral follicles
		1	2	3	4	5	6	
125–150	76 (5)	0	0	1.3	3.9	40.8	30.2	76.3[a)]
151–175	74 (8)	0	0	4.0	39.2	31.1	14.9	89.2[b)]

a, b) Values with different superscripts differ significantly ($p<0.05$).

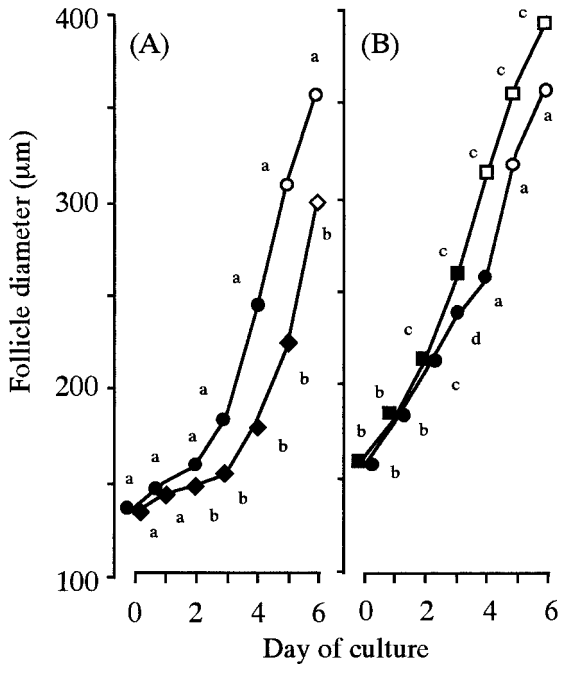

Fig. 1. Growth profiles of follicles of initial diameters of 125–150 μm (A) and 151–175 μm (B) grouped according to the day of antrum formation in each category. Data represent mean diameters on each day of culture. Different symbols represent the follicles which formed antra on day 4 (□, n = 34) , day 5 (○, n = 35 and n = 10 in A and B, respectively) and day 6 (◇, n = 31). Closed and open symbols represent preantral and antral follicle stages, respectively. Different letters (a-d) indicate significant differences ($p<0.05$) among the four subgroups on the same day of culture.

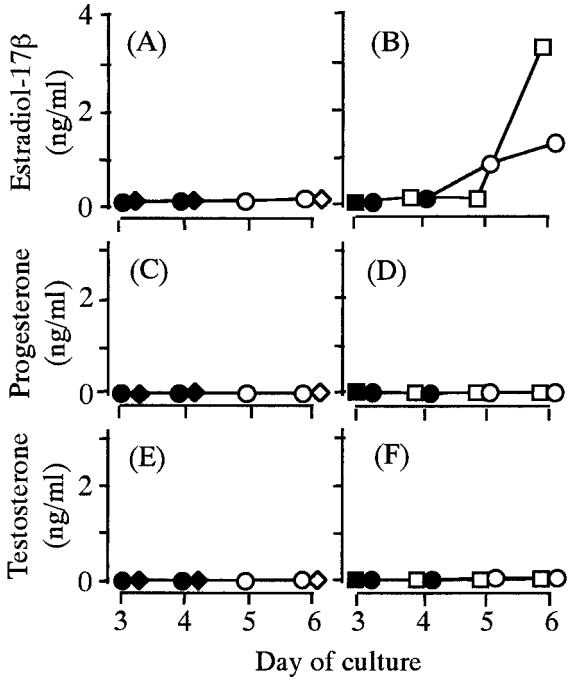

Fig. 2. Steroid production by 125–150 μm (A, C and E) and 151–175 μm (B, D and F) follicles which showed antrum formation on days 4 (□), 5 (○) and 6 (◇) *in vitro*. For closed and open symbols see Fig. 1. Data for day 5 for follicles forming antra on day 6 in A, C and E were not determined.

major populations of follicles and determined their growth and steroid production. Results are shown in Figs. 1 and 2. Small (125–150 μm) follicles which formed antra on days 5 or 6, showed lower growth rates (5–13 μm/day) than larger (151–175 μm) follicles which formed antra on days 4 or 5 (19–32 μm/day) during the first 2 days of culture ($p<0.05$). Faster growing follicles showed antra earlier and a bigger diameter at the end of culture. However, there were no differences in the follicle diameters at the time of antrum formation (ranged between 298 and 323 μm, $p>0.05$) regardless of the differences in the starting size and the time of antrum formation (Fig. 1). The diameters of follicles which showed antra on day 5 in the 125–150 and 151–175 μm category were not different on the last 3 days of culture ($p>0.05$).

As shown in Figs. 2A and B, the 151–175 μm follicles forming antra on day 4 produced more estradiol-17β on day 6 of culture (3.3 *n*g/m*l*/day) than other follicles (0.1–1.2 *n*g/m*l*/day). Progesterone and testosterone production was low in both follicle categories (Fig. 2C-F).

Study 2: All *in vivo* grown follicular oocytes showed meiotic resumption, whereas some oocytes from *in vitro* grown follicles did not resume meiosis (Table 2). Percentages of M II oocytes in *in vitro* grown follicles of both categories were similar to those of *in vivo* grown follicles. Oocytes from *in vitro* grown follicles showed lower ($p<0.01$) developmental rates than those from *in vivo* grown follicles (Table 3). Although blastocysts were only obtained

Table 2. *In vitro* maturation of oocytes derived from *in vitro* and *in vivo* grown follicles

Follicles (oocyte source)	Initial size (μm)	No. of COC for IVM (replicates)	% of oocytes at each stage			% of oocytes degenerated
			GV	GVBD	M II	
In vitro grown	125–150	53 (4)	20.8[a)]	17.0	62.3	0
	151–175	43 (3)	16.3[a)]	11.6	69.8	2.3
In vivo grown	–	138 (3)	0[b)]	26.1	73.9	0

COC = cumulus-oocyte complex, GV = intact germinal vesicle, GVBD = germinal vesicle breakdown, M II = oocytes with first polar body.
a, b) Values with different superscripts within same column differ significantly ($p<0.01$).

Table 3. *In vitro* development of oocytes derived from *in vitro* and *in vivo* grown follicles following *in vitro* fertilization

Follicles (oocyte source)	Initial size (μm)	No. of oocytes inseminated (replicates)	% of oocytes developed to				
			2-cell (24 hr)*	4-cell (48 hr)*	morula (72 hr)*	blastocyst (96 hr)*	hatched blastocyst (120 hr)*
In vitro grown	125–150	46 (2)	34.8[a)]	23.9[a)]	13.0[a)]	0[a)]	0[a)]
	151–175	23 (2)	30.4[a)]	21.7[a)]	13.0[a)]	13.0[b)]	8.7[a)]
In vivo grown	–	113 (3)	88.5[b)]	77.0[b)]	85.0[b)]	69.9[c)]	69.9[b)]

* Hours after insemination *in vitro*.
a-c) Values with different superscripts within same columns differ significantly ($p<0.05$).

from the oocytes of the 151–175 μm category, oocytes from both categories developed to the morula stage.

DISCUSSION

In the present study, differences in the growth and antrum formation rates, and the timing of antrum formation between the two categories were closely related to the size of the follicles at the start of culture. These findings were expected, since a majority of the small (125–150 μm) and large (151–175 μm) follicles were estimated as types 4 and 5a, respectively, according to the classification by Pedersen and Peters [18]. Estimated follicle growth *in vivo* is characterized as slow for type 4 follicles and faster in subsequent types 5a and 5b towards the antral stage (type 6) [17]. The small follicles (type 4) showed slow growth during the first 2 days of culture, and a subsequent higher growth rate towards antrum formation, whereas the large follicles estimated as type 5a showed relatively high growth rates from the beginning of culture. The follicle growth patterns under the present culture conditions appear to be similar to those *in vivo*.

Supplementation of follicle culture medium with FSH was reported to be necessary for antrum formation of mouse preantral follicles *in vitro* [3]. Therefore, we used a medium supplemented with 0.1 IU/m*l* of FSH to maximize follicular growth [16]. According to the follicular growth *in vivo* [17], types 4 and 5a follicles can take 4–8 and 3–4 days to reach type 6, respectively. This might be one of the reasons for the differences in the antrum formation rates between the small and large follicle categories. In the present study, we cultured the preantral follicles of both categories for 6 days and did not determine antrum formation beyond day 6.

The majority of follicles of both categories formed antra when they attained a diameter of 300–320 μm in the present medium supplemented with FSH. These results are consistent with previous reports [3, 13, 14] in which follicles with initial sizes of 140–250 μm formed antra when they attained diameters of 300–330 μm in media supplemented with mouse serum and FSH.

Based on the timing of antrum formation, two major subgroups of follicles were observed in each category of this study: follicles that showed antra on days 5 and 6 in the small follicle category, and on days 4 and 5 in the large follicle category. In the present study, most of the follicles with initial diameters of 125–150 μm formed antra on days 5 and 6, but could not reach the preovulatory size (≥380 μm) [3] within 6 days of culture. This can be attributed to the differences in the follicle stages at the start of culture within each category: such as early or late phase of types 4 and 5a.

Oocytes from adult mice acquire meiotic competence to reach M II soon after follicular antrum formation [9]. Moreover, supplementation of maturation medium with EGF has been reported to enhance M II competence of oocytes

derived from *in vitro* and *in vivo* grown follicles [2, 4]. We cultured COCs using a maturation medium supplemented with EGF so that the oocytes from *in vitro* grown follicles of two categories might show similar M II rates to those from *in vivo* grown follicles.

Embryonic development of oocytes derived from *in vitro* grown preantral follicles has been reported in an individual/spherical follicle culture system [19, 20]. Cleavage rates of *in vitro* fertilized oocytes derived from the present *in vitro* grown follicles of both categories (30 and 35%) were similar to those from *in vitro* grown follicles with 190 ± 10 μm of starting size in a previous study [20].

The capacity of oocytes to develop to the blastocyst stage has been shown to be dependent on the size [6] and ability of estradiol-17β production [1] of the antral follicles from which the oocytes were isolated. The frequency of 2-cell stage mouse embryos that developed to the blastocyst stage was lower in the small (240–320 μm) follicles than in the large (360–400 μm) follicles [7]. Bovine oocytes derived from the antral follicles with lower estradiol-17β production had lower developmental competence to the blastocyst stage [1]. Furthermore, most of the 2-cell stage mouse embryos derived from ovulated ova reached the blastocyst stage; whereas, progressively fewer embryos derived from the oocytes of *in vitro* grown follicles were able to advance past the 2-cell stage to the morula stage and the blastocyst stage [6]. These previous findings indicate that the capacity of oocytes to develop to the blastocyst stage is acquired when they complete both nuclear and cytoplasmic maturation during the period of growth to the preovulatory stage. In the present study, about half of preantral follicles of the large category reached the preovulatory size and increased estradiol-17β production before their oocytes were subjected to maturation culture, so some of their oocytes could develop to the blastocyst stage. Most of the follicles of the small category, however, did not attain the preovulatory size and showed no increase in estradiol-17β production at the end of culture, so few oocytes could obtain the developmental capacity to reach the blastocyst stage. These results suggest that small follicles should be cultured another 1 or 2 days before their oocytes are subjected to the maturation culture to give them the competence to develop to the blastocyst stage.

The present culture system is a modification of the original system described by Boland *et al.* [3] that produced pups from 190 ± 10 μm sized follicles [20]. FSH is a major supplement to the growth medium. Growth of small preantral mouse follicles with 2–3 layers of granulosa cells (type 4 and 5a), the target of the present study, is influenced by basal levels of FSH [11]. Further, antrum formation appears to be FSH-dependent in cultured mouse follicles [3]. Substitution of mouse serum with FCS is the major modification to the original culture system. Mouse serum used as the major protein source in culture medium was reported to enhance the development of follicles resulting in higher end sizes and a morphology much more similar to that observed *in vivo* compared to FCS [16]. In the present study, however, the majority of follicles remained intact throughout the culture period, and the growth pattern appeared to be similar to that *in vivo*. These results indicate that a follicle culture medium supplemented with FSH and FCS can support preantral follicle development to the preovulatory stage.

In conclusion, the present results demonstrate that the individual/spherical follicle culture system using a medium with FSH and FCS could support *in vitro* growth of mouse preantral follicles with diameters of 151–175 μm to the preovulatory stage, and that their oocytes acquire the capacity to develop to the blastocyst stage. However, further study is needed to determine optimal culture conditions for the development of preantral follicles and the developmental capacity of their oocytes to normal pups.

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