**In vitro** Culture Conditions for the Mouse Preantral Follicles Isolated by Enzyme Treatment

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**ABSTRACT:** In order to investigate the factors affecting the culture of mouse preantral follicles *in vitro*, we examined the effect of culture media, protein supplements, and culture period on their growth. The oocyte diameter (initial size: 55.6±2.5 μm) was progressively increased during culture, and the maximum size (72.0±2.4 μm) was reached on day 10 of the *in vitro* culture. The chromatin configuration in the germinal vesicle (GV) oocyte progressively shifted from a non-surrounded nucleolus (NSN) to a surrounded nucleolus (SN). On day 10 of the culture, most of the oocytes progressed to the SN pattern. The survival and metaphase II rates of the oocytes in alpha-minimal essential medium (alpha-MEM) were significantly higher (p<0.05) than those in Waymouth and tissue culture medium (TCM)-199. As a protein source, fetal bovine serum (FBS) was more suitable for the culture of mouse preantral follicles as compared to human follicular fluid (hFF) and bovine serum albumin (BSA); the optimal concentration of FBS was 5%. These results suggest that in a culture of mouse preantral follicles, alpha-MEM and 5% FBS are an optimal medium and a protein source, respectively; further, the 10 days of culture is required for the complete growth of oocytes in this culture system. (**Key Words**: Chromatin Pattern, Media, Mouse Preantral Follicle, Protein)

**INTRODUCTION**

Recent advances in *in vitro* reproductive technologies have opened up new opportunities in assisted reproduction technology (ART) and biotechnology. However, these techniques depend on the predictable production of fully developed oocytes, and currently, their availability is limited by the number of antral follicles present in the ovaries. The development of a preantral follicle culture system that can potentially produce large quantities of oocytes with uniform developmental status will significantly advance the use of these techniques. Additionally, it might enable the preservation and long-term storage of the female germ cells (Gutierrez et al., 2000).

Various methods have been developed to isolate and culture preantral follicles from mouse ovaries (Eppig and Schroeder, 1989; Cortvrindt et al., 1996). In general, complex media have been used for the culture of preantral follicles. A large variety of additives, such as serum or serum supplements and growth factors, have been employed for medium supplementation. Under appropriate conditions, the meiotically incompetent oocytes from the preantral follicles can grow to their final size and complete nuclear maturation *in vitro*. Furthermore, studies regarding the culture of mouse preantral follicles have demonstrated successful growth and maturation as well as the fertilization and development of oocytes from follicles cultured *in vitro* (Eppig and Downs, 1989; Nayudu and Osborn, 1992; Cortvrindt et al., 1996; Kim et al., 2004). However, synthetic studies concerning the culture conditions of mouse preantral follicles *in vitro* have not been accomplished.

In order to establish a suitable culture condition for the mouse preantral follicles that were enzymatically isolated from the ovary, we conducted a series of experiments with the following aims: (1) to determine the optimal culture period of preantral follicles according to the change in oocyte diameter and chromatin configuration; (2) to compare the effects of the culture medium and protein sources on the survival and maturation rates of preantral follicles *in vitro*; and (3) to examine the optimal concentration of fetal bovine serum (FBS) for the growth
Table 1. Change in the oocyte diameter according to the culture period

<table>
<thead>
<tr>
<th>Day</th>
<th>Day 0</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 6</th>
<th>Day 8</th>
<th>Day 10</th>
<th>Day 12</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oocyte diameter</td>
<td>55.6±2.8</td>
<td>59.3±3.5</td>
<td>62.4±3.1</td>
<td>66.0±3.0</td>
<td>70.2±2.1</td>
<td>72.0±2.4</td>
<td>71.0±2.2</td>
<td>72.3±4.0</td>
</tr>
<tr>
<td>(SEM, μm)</td>
<td>(57)*</td>
<td>(54)*</td>
<td>(61)*</td>
<td>(53)*</td>
<td>(56)*</td>
<td>(55)*</td>
<td>(57)*</td>
<td>(67)*</td>
</tr>
</tbody>
</table>

* The number of oocytes examined.

and development of the preantral follicles in vitro.

MATERIALS AND METHODS

Isolation of preantral follicles

The ovaries were aseptically removed from the 12-day-old ICR female mice. The ovaries were immersed into Leibovitz L-15 medium (Gibco-BRL, Carlsbad, CA, USA) containing 1 mg/ml collagenase (Type 1A; Sigma, St. Louis, MO, USA) and 0.2 mg/ml DNase I (Sigma) for 20 min at 37°C and repeatedly drawn in and out of the pipette until the ovaries were dissociated into individual follicles. The preantral follicles (100-120 μm in diameter) to be cultured were selected based on the following criteria: i) intact round follicular structure with 2-3 layers of granulosa cells and ii) the oocyte had to be visible, round, and centrally located within the follicle. All the selected follicles were pooled and randomly divided over the culture conditions under study.

In vitro growth and maturation of the preantral follicles

The culture medium was supplemented with a protein supplement and 100 mLU/ml follicle stimulating hormone (FSH, Metrodin-HP; Sereno, Switzerland) and 10 mLU/ml luteinizing hormone (LH; Sigma). The follicles were cultured on Transwell-COL membrane inserts (3.0 μm pore size, 24.5 mm diameter; Costar, NY, USA) in 6-well cluster dishes to prevent the loss of structural integrity between the oocyte and the granulosa cells. The follicles were cultured for 10 or 14 days at 37°C in 5% CO₂ in air. Half of the medium was changed every 2 days. After 10 days of growth in vitro, the follicles were allowed to mature for 16-18 h in a medium supplemented with 1.5 IU/ml human chorionic gonadotrophin (hCG, Profasi; Sereno). The diameter of the morphologically normal metaphase II oocytes, excluding the zona pellucida, was examined with an inverted microscope and an ocular micrometer.

Preparation of human follicular fluids

During oocytes aspiration in the human IVF programs, human follicular fluids (hFF) were collected from the preovulatory follicles of the patients. The blood cells and cell debris were removed from the follicular fluids by centrifugation at 3,000 rpm for 30 min. The supernatant was heat-inactivated at 59°C for 35 min and filtered with a 0.22-μm microfilter (Milllex-GV; Millipore, Bedford, MA, USA). All the hFF samples were stored at -70°C.

Nuclear chromatin staining

The chromatin configuration of the germinal vesicle (GV) oocytes during culture was evaluated by fluorescence staining. The oocytes were fixed in 2% formaldehyde in phosphate buffered saline (PBS) for 10 min at room temperature. The fixed oocytes were then placed on slides with a drop of the mounting medium consisting of glycerol and PBS containing 2.5 mg/ml sodium azide and 2.5 μg/ml Hoechst 33342 (Sigma); glycerol and PBS were in the ratio of 3:1. A coverslip was placed on top of the oocytes, and the edge was sealed with fingernail polish. The stained oocytes were examined under a fluorescence microscope.

Experimental designs

Experiment 1: To determine optimal culture period for mouse preantral follicles, the mouse preantral follicles were cultured in α-minimal essential medium (αMEM) supplemented with 5% FBS, 100 mLU/ml FSH, and 10 mLU/ml LH for 14 days. The assessment of the oocyte diameter and nuclear configuration was carried out by the mechanical removal of the oocytes from their follicular complex on days 2, 4, 6, 8, 10, 12, and 14.

Experiment 2: To compare the effects of different culture media (αMEM, Waymouth, and tissue culture medium (TCM)-199) and protein sources (FBS, hFF, and bovine serum albumin (BSA)) on the survival and maturation rates of the preantral follicles in vitro, the mouse preantral follicles were cultured for 10 days and further cultured in a medium supplemented with 1.5 IU/ml hCG for 16-18 h to induce meiotic maturation.

Experiment 3: To examine the effect of the various concentrations of FBS (5, 10, 15, and 20%) on the preantral follicle growth and development, the mouse preantral follicles were cultured in αMEM as described in experiment 2.

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 the Duncan’s multiple range tests. Statistical significance was established at p<0.05.

RESULTS

Change of oocyte diameter during the culture period

The change in oocyte diameter within the preantral
The mean oocyte diameter at the beginning of the culture was 55.6 ± 2.5 μm. During the culture period, the oocyte diameter increased progressively. The maximum size (72.0 ± 2.4 μm) of the oocytes was reached at 10 days after culture. On day 10 of the in vitro culture, most of the oocytes progressed from the NSN to the SN pattern, and this pattern change was correlated with the increase in oocyte diameter as shown in Table 1.

**Effect of culture medium**

In order to determine the optimal culture medium, the mouse preantral follicles were cultured in 3 different culture media. As shown in Table 2, the survival and metaphase II rates of the preantral follicles following in vitro growth and maturation was significantly higher in αMEM (68.3% and 44.5%, respectively) than in Waymouth (54.9% and 15.9%, respectively) and TCM 199 (39.0% and 15.2%, respectively). With respect to the mean diameter of the matured oocytes, there was no difference among those cultured with αMEM (67.7±0.9 μm), Waymouth (69.3±1.1 μm) and with TCM 199 (70.0±1.1 μm).

**Effect of protein sources**

In order to examine the optimal protein sources, the
Table 3. Effect of protein sources on the in vitro growth and maturation of mouse preantral follicles

<table>
<thead>
<tr>
<th>Protein</th>
<th>No. of follicles cultured</th>
<th>No.(%) of oocytes survived*</th>
<th>GV (%)</th>
<th>GVBD (%)</th>
<th>Meta II (%)</th>
<th>Oocyte diameter (SEM, μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% FBS</td>
<td>197</td>
<td>147 (71.6)</td>
<td>49 (24.9)</td>
<td>20 (10.2)</td>
<td>72 (36.5)</td>
<td>67.3±2.1</td>
</tr>
<tr>
<td>5% hFF</td>
<td>197</td>
<td>130 (66.0)</td>
<td>59 (29.9)</td>
<td>41 (20.8)</td>
<td>30 (15.2)</td>
<td>69.5±1.6</td>
</tr>
<tr>
<td>0.3% BSA</td>
<td>197</td>
<td>145 (73.6)</td>
<td>112 (56.9)</td>
<td>32 (16.2)</td>
<td>10 (5.1)</td>
<td>70.4±2.0</td>
</tr>
</tbody>
</table>

GV = Germinal 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.

** Significant differences within the same column (p<0.05).

Table 4. Effect of FBS concentrations on the in vitro growth and maturation of mouse preantral follicles*

<table>
<thead>
<tr>
<th>Conc. (%)</th>
<th>No. of follicles cultured</th>
<th>No.(%) of oocytes survived**</th>
<th>GV (%)</th>
<th>GVBD (%)</th>
<th>Meta II (%)</th>
<th>Oocyte diameter (SEM, μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>148</td>
<td>90 (60.8)</td>
<td>19 (10.7)</td>
<td>15 (8.4)</td>
<td>56 (37.8)</td>
<td>69.2±2.9</td>
</tr>
<tr>
<td>10</td>
<td>147</td>
<td>95 (64.6)</td>
<td>29 (19.7)</td>
<td>15 (10.2)</td>
<td>51 (34.7)</td>
<td>68.8±2.7</td>
</tr>
<tr>
<td>15</td>
<td>148</td>
<td>71 (48.0)</td>
<td>20 (13.5)</td>
<td>4 (2.7)</td>
<td>47 (31.8)</td>
<td>68.4±2.6</td>
</tr>
<tr>
<td>20</td>
<td>147</td>
<td>71 (48.3)</td>
<td>12 (8.2)</td>
<td>13 (8.8)</td>
<td>46 (31.3)</td>
<td>69.0±1.8</td>
</tr>
</tbody>
</table>

GV = Germinal vesicle, GVBD = Germinal vesicle breakdown, Meta II = Metaphase II.
* There were no significant differences among the four concentration treatments in oocyte survival and metaphase II rates and oocyte diameter.
** 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.

Effect of FBS concentration

To examine the optimal concentration of FBS on the culture of mouse preantral follicles, the preantral follicles were cultured with various concentrations of FBS. As shown in Table 4, the survival rates of the preantral follicles following in vitro growth and maturation were higher at 5% and 10% concentrations (60.8% and 64.6%, respectively) than at 15% and 20% concentrations (48.0% and 48.3%, respectively), but there was no statistical significance. The metaphase II rates of the oocytes were slightly higher at 5% concentration (37.8%) than at 10%, 15% and 20% (34.7%, 31.8% and 31.3%, respectively), although there was no statistical significance among the concentration of FBS. The mean diameter of the matured oocytes did not differ among the treatment groups.

**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 1-3 layers of granulosa cells around the oocyte. Such oocytes are approximately in a mid-growth phase and are incompetent to undergo a germinal vesicle breakdown (GVBD) at the time of isolation (Eppig and Downs, 1989); however, the follicular cells possess all the morphological and functional abilities to sustain follicular growth. In this experiment, we also confirmed that the oocytes were incapable of resuming meiosis at the time of isolation from preantral follicles.

Based on the purpose of the experiment, various culture methods for the growth of 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 microtiter plates (Hartshorne et al., 1994; Spears et al., 1994), and microdroplets covered with mineral oil (Cortvrindt et al., 1996; Choi et al., 2007). In the present study, we used collagen-coated Transwell-COL membrane inserts (Eppig and Schroeder, 1989) to culture mouse preantral follicles. These membranes were treated with an equimolar mixture of Type I and III collagen produced from bovine placenta, and they allowed the attachment and maintenance of the complexes with only minimal migration of the granulosa cells from the oocytes as compared to the general petri dishes. Generally, it is more difficult to maintain a three-dimensional structure between the oocyte and granulosa cells in the enzymatically isolated preantral follicles because theca cells and the basal lamina are partially damaged by collagenase. However, the culture of preantral follicles on the Transwell-COL membrane makes it possible for the oocyte and granulosa cells to maintain the complex...
as a three-dimensional structure that helps in the survival and growth of oocytes and follicles.

The diameter of the oocyte is an important index for the cytoplasmic maturation and developmental competence of oocytes. In the present study, the mean oocyte diameter at the start of culture was 55.6±2.5 μm and progressively increased during the culture period. The maximum diameter (72.0±2.4 μm) of the oocytes was reached at 10 days after culture. The diameter of these oocytes was comparable to that of the oocytes (73.3±3.0 μm) from 22-day-old mice (after ovarian stimulation and in vitro maturation) (Kim et al., 2004). Thus, the results from the present study confirmed that in this culture condition, the optimal culture period for the growth of mouse preantral follicles is 10 days.

Earlier studies have shown that 2 different classes of oocytes are present within the antral compartment of the mouse ovary (Mattson and Albertini, 1990; Debej et al., 1993; Zuccotti et al., 1995). One class known as SN is characterized by the presence of a ring of Hoechst-positive chromatin around the nucleolus and a thread-like nuclear chromatin organization; the other class NSN has more homogeneously widespread nuclear chromatin and less well-defined chromatin surrounding the nucleolus. Several observations suggest that the SN configuration is a prerequisite for GVBD and is a stage of the GV oocyte that may be useful not only investigate the critical questions of follicular development, but also give assistance on the future management of conservation in endangered and rare animals.

REFERENCES


