In Vitro Growth, Maturation, Fertilization, and Embryonic Development of Oocytes from Porcine Preantral Follicles

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ABSTRACT

This study was conducted to identify an in vitro culture system that would support intact porcine follicle growth from preantral follicle to antral stages, oocyte maturation, fertilization, and embryonic development; and to evaluate factors that influence porcine preantral follicle growth in vitro. Preantral follicles isolated from prepubertal porcine ovaries were cultured for 4 days in the presence of different concentrations of porcine serum and FSH, and with different numbers of follicles per well. A series of experiments showed that porcine antral follicles can be grown at a high frequency in vitro from healthy preantral follicles with intact theca when cultured in North Carolina State University 23 medium supplemented with 1.5 ng/ml FSH, 7.5% serum, and when cultured with three follicles per well. After 4 days of culture, 68% healthy cumulus-enclosed oocytes from these follicles were obtained, and 51% of the oocytes completed meiotic maturation to the metaphase II stage. Fifty-three percent of the mature oocytes underwent fertilization, 43% of the fertilized oocytes cleaved, and 13% developed to the blastocyst stage. The results show 1) that porcine preantral follicles can grow efficiently to the antral stage using these culture conditions, and 2) that oocytes from in vitro-matured porcine preantral follicles can acquire meiotic competence and undergo fertilization and embryonic development.

INTRODUCTION

The ovarian follicle is the structural and functional unit of the mammalian ovary. It consists of an oocyte, its surrounding granulosa cells, a basement membrane, and associated thecal cells arranged adjacent to the basement membrane [1]. Normal follicular development of an oocyte that is capable of fertilization and embryonic development depends upon a complex sequence of cellular interactions within the follicle. These interactions create a unique and progressively shifting environment during the development of the oocyte. At any stage during this development, the follicle may continue normally or, more frequently, proceed toward atresia.

Current methods for the in vitro production of embryos are dependent upon a supply of developmentally competent oocytes from large antral or preovulatory follicles, which are present in the ovary only in relatively small numbers. However, mammalian ovaries contain a number of primordial and preantral follicles. Applications for the use of in vitro-matured preantral follicles include the animal production system, transgenesis research, conservation of rare breeds, and as a potential source of genetic material [2–6].

Studies involving isolation of ovarian follicles and analysis of the requirements, metabolism, and differentiation processes of in vitro maturation of ovarian follicles have already been undertaken by many researchers using rodents as models [7–20]. In contrast, the complete development in vitro of preantral follicles obtained from human or domestic animal ovaries has been more difficult to achieve [21–24]. This is due to the greater follicle dimension and to the presence of a thick theca, which restricts the transport of nutrients and gases during the long-term culture period required for follicle culture.

The aim of this study was 1) to identify an in vitro culture system capable of supporting intact porcine follicle growth from the preantral to antral stages, then through oocyte maturation, fertilization, and embryonic development; and 2) to evaluate factors that influence porcine preantral follicle growth in vitro.

MATERIALS AND METHODS

Animals

Ovaries from prepubertal gilts were collected at a local abattoir and rinsed in Dulbecco’s PBS (PDBS; Gibco 11500-030, Grand Island, NY) supplemented with 3 mg/ml BSA (A 8022 fraction V, Sigma, St. Louis, MO). The ovaries were kept at 37°C during 45–60 min of transportation from the slaughterhouse to the laboratory. Blood from prepubertal gilts was collected and centrifuged for 10 min. The serum was collected and stored at −20°C.

Preantral Follicle Collection and In Vitro Culture

The ovaries were washed with DPBS and transferred into DPBS with 3 mg/ml BSA, and cut into small pieces (1–3 mm) with a blade. Preantral follicles were isolated by forceps dissection. Follicles with a visible oocyte, homogenous granulosa layers, and thecal cells in the size range of 200–310 μm were collected into 24-well multidishes (Nunclon) containing holding medium (North Carolina State University 23 medium [NCSU23]) [25] supplemented with 3 mg/ml BSA (A 8022 fraction V, Sigma). After follicles were collected they were transferred from the holding medium into the culture medium. The culture medium consisted of NCSU23 supplemented with 3.5 μg/ml insulin (I 5523, Sigma); 10 μg/ml transferrin (T 5391, Sigma); 100 μg/ml L-ascorbic acid (A 4544, Sigma); and 5%, 7.5%, or 10% porcine serum, depending on the experiment. Except for control groups, the culture medium was also supplemented with ovine FSH (oFSH-20, 4453 IU/ml, the National Hormone and Pituitary Program of National Institute of Diabetes and Digestive and Kidney Diseases [NIDDK], Torrance, CA) in different concentrations on the basis of the experiment design.

Follicles within the size ranges just described were ran-
dominantly distributed to different experimental groups. Depending on the experimental group, 24-well cell culture clusters plates (3524, Costar, Corning, NY) with one, three, or five follicles were cultured in each well containing 280 μl of culture medium for 4 days. The culture was carried out at 38.5°C and gassed with 5.0% CO₂ in air. The 4-day culture period was chosen after preliminary studies showed that a period longer than 4 days had a deleterious effect on follicle cell survival under the culture conditions described above and based on the studies of Fehrenbach et al. [26] and Ceconi et al. [23]. The diameters of follicles (including theca layer) were measured every 24 h using a stereomicroscope ocular scale at a magnification of 50×. The culture medium was changed every 2 days with freshly prepared culture medium.

### In Vitro Maturation of Oocyte-Cumulus Complexes

Maturation of oocyte-cumulus complexes (OCCs) was performed as described by Wu et al. [20] with modifications. Follicles were transferred to DPBS supplemented with 3 mg/ml BSA at the end of culture, and were then carefully opened. After washing three times in holding medium supplemented with 0.23 mM pyruvate and 10% porcine serum, OCCs were transferred to drops of the same medium that also contained 0.12 mM caffeine and 2 mg/ml BSA (A 7888, Sigma) and modified Krebs-Ringer bicarbonate (mKRB) solution [25], respectively. Washed spermatozoa were diluted to 2 × 10⁸ cells/ml in the mKRB solution and incubated for 90 min at 38.5°C in an atmosphere of 5% CO₂ in air [29]. After an appropriate dilution with IVF medium, 50 μl of this sperm suspension was added to 50 μl of the medium that contained oocytes (final concentration of 5 × 10³ cells/ml). Between 5 and 6 h after insemination, oocytes were removed from fertilization drops and cultured in 500 μl of embryo culture medium (NCSU23 containing 4 mg/ml BSA) in a 60 × 15-mm² center-well organ culture dish (Becton Dickinson, Franklin Lakes, NJ) until examination. At 48 and 192 h after insemination, cleavage rate and blastocyst formation were evaluated under a stereomicroscope. Fertilization rates were calculated by adding the cleaved embryos and one-cell oocytes that had been penetrated by spermatozoa. One-cell oocytes were mounted on slides at 48 h after insemination. The oocytes were fixed in 25% (v/v) acetic alcohol, stained with 1% (w/v) orcein in 45% (v/v) acetic acid, and examined under a phase-contrast microscope for penetration by a sperm and pronuclear development.

### Experimental Design

To identify the optimal conditions supporting intact porcine preantral follicle growth in vitro, experiment 1 was carried out using an orthogonal design (orthogonal array, L₉(3⁴)) [30] consisting of 1) number of follicles per well (1, 3, 5), 2) serum (5%, 7.5%, 10%), and 3) FSH (0.375, 0.75, 1.5 ng/ml; Table 1). A total of 18–21 follicles were cultured in each of the nine conditions, for an overall total of 175 follicles in the experiment. Each condition was repeated four to six times. Based on experiment 1, experiment 2 was carried out to evaluate the effects of FSH, serum, and number of follicles per well on follicle growth patterns in vitro (total of 11 treatment groups). Each treatment was repeated at least four times. A total of 412 follicles were cultured in the experiment.

In experiment 3, the developmental competence of oocytes from follicles cultured under the optimal conditions was evaluated. A total number of 244 preantral follicles

### Table 1. Preantral follicle growth under the different conditions.a

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Number of follicles per well</th>
<th>Serum (%)</th>
<th>FSH (ng/ml)</th>
<th>Number of cultured follicles</th>
<th>Follicle diameterb (mean ± SEM μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>5</td>
<td>0.375</td>
<td>18</td>
<td>252 ± 9.6, 365 ± 12.2</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>7.5</td>
<td>0.75</td>
<td>19</td>
<td>248 ± 10.7, 401 ± 9.5</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>10</td>
<td>1.5</td>
<td>18</td>
<td>253 ± 9.3, 428 ± 9.3</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>5</td>
<td>0.375</td>
<td>21</td>
<td>250 ± 9.8, 374 ± 10.9</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>10</td>
<td>1.5</td>
<td>18</td>
<td>249 ± 11.1, 477 ± 9.9</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>10</td>
<td>0.375</td>
<td>21</td>
<td>251 ± 10.3, 371 ± 11.8</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>5</td>
<td>1.5</td>
<td>20</td>
<td>247 ± 8.8, 416 ± 11.2</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>7.5</td>
<td>0.375</td>
<td>20</td>
<td>250 ± 10.6, 369 ± 11.4</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>10</td>
<td>0.75</td>
<td>20</td>
<td>251 ± 8.9, 409 ± 12.5</td>
</tr>
</tbody>
</table>

* Each condition was repeated four to six times.
* The diameters between conditions were not different on Day 0.
* P < 0.05 as compared with other conditions.
were cultured and oocytes from these follicles were matured, fertilized, and developed as embryos in vitro. This treatment was repeated eight times.

**Statistical Analysis**

Only follicles that grew and remained intact for the entire culture period (4 days) were considered for analysis (>90%). One-way ANOVA was used for all statistical comparisons. The distributions of follicle starting size between experimental groups were not significantly different. *P* values of < 0.05 were considered significant.

**RESULTS**

**Establishment of the Optimal Conditions Supporting Intact Porcine Preantral Follicle Growth In Vitro**

In experiment 1, an orthogonal design of three factors at three levels was used to determine the optimal conditions supporting intact porcine preantral follicle growth in vitro. The preantral follicles were cultured under nine conditions for 4 days. Condition 5 (3 follicles per well, 7.5% serum, and 1.5 ng/ml FSH) produced a final follicle size that was significantly larger (477 ± 9.9 μm) than was achieved with other conditions (≤428 μm, Table 1), and induced antral formation in 89% of follicles. It was therefore concluded that three follicles per well, 7.5% serum, and 1.5 ng/ml FSH can best support preantral follicle growth in vitro.

**Effect of Porcine Serum Concentration on Preantral Follicle Growth Patterns**

The effect of 5%, 7.5%, and 10% porcine serum on the growth patterns of preantral follicles was examined under optimal FSH concentration and three follicles per well. Shown in Figure 1A are the comparative effects of serum concentration in the presence of 1.5 ng/ml FSH and three follicles per well. The 7.5% porcine serum produced a final follicle size that was significantly larger than was achieved with 5% serum or 10% serum. The 5% serum supported rapid growth (58 μm/day) for only the first 24 h; thereafter, the growth rate reduced to an average of 22 μm/day and an end size of 377 ± 10.9 μm (n = 38) was produced. An increase of serum concentration to 7.5% (together with optimal FSH concentration and three follicles per well) appeared to produce a potentiating effect and resulted in the maintenance of rapid growth for the first 3 days of culture with a growth rate of 67 μm/day. This resulted in a mean final diameter of 479 ± 9 μm (n = 40) with a high proportion (88%) of antral formation. In contrast, a further increase to 10% serum sustained rapid growth for only 2 days, resulting in a mean endpoint diameter of 414 ± 11.6 μm (n = 36), indicating an overdose effect.

**Effect of FSH on Preantral Follicle Growth Patterns**

Growth of porcine preantral follicles was tested by culturing in the absence (control) or in the presence of increasing FSH concentrations with 7.5% serum and three follicles per well to determine the effect of FSH on follicle growth patterns (Fig. 1B). The zero FSH control (with 7.5% serum and three follicles per well) produced rapid growth for the first 24 h, but this growth was not sustained, and the final mean follicle size was only 350 ± 9 μm (n = 34). An FSH concentration of 0.375 ng/ml produced a significant increase in growth, but the follicles did not reach the antral threshold size (average growth rate 32 μm/day, mean end

![FIG. 1.](https://academic.oup.com/biolreprod/article-abstract/64/1/375/2723830)
FIG. 2. Examples of living follicles from start size to antrum formation, and healthy cumulus-enclosed oocytes. A) Preantral follicle on Day 0 of culture. B) Antral follicle on Day 4 of culture. C) Morphologically normal oocyte enclosed cumulus mass. Bar = 50 μm.

Effect of Number of Follicles Per Well on Preantral Follicle Growth Patterns

In the presence of 1.5 ng/ml FSH and 7.5% serum, an important factor in follicle growth was the number of follicles in the well. The effect produced by number of follicles per well is shown in Figure 1C, where three follicles per well produced a significantly larger final size of follicles (479 ± 9.5 μm, n = 46) than was achieved with one follicle per well (408 ± 11.2 μm, n = 40) or five follicles per well (420 ± 6.3 μm, n = 42). This was most apparent over the first 2 days of culture when there was a growth rate of 101 μm/day for the first 24 h and, thereafter, continued to grow at a rate of 59 μm/day. During the first 2 days, one follicle per well induced a significantly less increase in diameter (74 μm/day for Day 1, 33 μm/day for Day 2) than three follicles per well. Five follicles (97 μm/day for first 24 h) and three follicles per well induced similar growth rates during the first day of culture. In cultures with five follicles per well, however, the growth rate was not sustained during the second 24 h, and the increase of follicle diameter was significantly less (30 μm/day) than with three follicles per well.

In Vitro Growth, Maturation, Fertilization, and Embryonic Development of Oocytes from Preantral Follicles

Under the optimal culture conditions (1.5 ng/ml FSH, 7.5% serum, and three follicles per well), 244 preantral follicles (starting size, 245 ± 11.7 μm, Fig. 2A) grew rapidly to a final size of 480 ± 8.1 μm (average growth rate 59 μm/day) with a high percentage (87%) of antrum formation (Fig. 2B) after 4 days in culture. At the end of the 4-day period, each follicle was assessed for quality of the related OCC and then OCC maturation. The percentage of healthy OCCs with morphologically normal oocytes enclosed by intact cumulus cells (Fig. 2C) was 69%. Oocyte maturation rate was 51% (124 of 244, Fig. 3A). Of the 124 mature oocytes, 10 were observed for chromatin and spindle configuration. Figure 3, B and C, are examples of chromatin and spindle configuration after in vitro maturation of oocytes from follicles cultured with the optimal conditions. The remainder (114) were inseminated. As shown in Table 2, 53% (60 of 114) of the oocytes were fertilized; 43% (26 of 60) by 48 h after IVF (Fig. 4A), and 13% (8 of 60) developed to the blastocyst stage after 192 h of culture (Fig. 4B).

DISCUSSION

This is the first report of in vitro embryonic development of oocytes from preantral follicles of a large animal species. Using optimized conditions, this study has demonstrated that porcine antral follicles can be grown at a high frequency in vitro from healthy preantral follicles with intact theca. After 4 days of culture, healthy cumulus-enclosed oocytes have been obtained, and half of the oocytes from the preantral follicles can complete meiotic maturation up to metaphase II stage. Moreover, 53% of the mature oocytes underwent fertilization, 43% of the fertilized oocytes cleaved, and 13% of them developed to the blastocyst stage.

Cecconi [23] reported that sheep preantral follicles with intact theca isolated by mechanical dissection of the ovary can grow to become antral follicles, and that some oocytes from them matured in vitro with a small volume of medium (25 μl) as well as in the presence of 10% serum and 1 μg/ml FSH. This is in agreement with our observation that
porcine preantral follicles with intact theca can grow to become antral follicles, and that oocytes from them can mature under the suitable conditions. However, this is different from other the system in which porcine preantral follicles without thecal cells isolated enzymatically grew to antral follicles and some oocytes from them matured by culturing in collagen gel, in the presence of serum, FSH, and estradiol [21].

Traditionally, complete optimization (optimal culture condition) can be achieved if each variable component is tested independently. For example, an analysis by full factorial design of three variables studied at three levels requires an experiment with 27 (3³) separate trials. However, because of simplicity and proven success in industrial process design, the orthogonal designs have recently offered a cost-effective strategy for the study of interactions between reaction variables in DNA amplification [31, 32]. The orthogonal designs provide accurate results and, as such, they have been widely used in the automotive and electronics industries with success and are now being extended to the food and pharmaceutical sectors, and other fields [22, 33, 34]. Orthogonal designs can also examine many factors in only a few experimental trials. In the present study, sets of three variables were arranged in an orthogonal design that defines only nine experimental trials. The conditions, which consisted of three follicles per well, 7.5% serum, and 1.5 ng/ml FSH, were determined as the optimal conditions for porcine preantral follicle development in vitro. The result was further clarified and detailed by evaluating the effects

<table>
<thead>
<tr>
<th>Number of oocytes examined</th>
<th>Number of oocytes fertilized (%)</th>
<th>≥2 cells (%)</th>
<th>≥3–16 cells (%)</th>
<th>Blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>114</td>
<td>60 (53)</td>
<td>26 (43)</td>
<td>18 (30)</td>
<td>8 (13)</td>
</tr>
</tbody>
</table>

FIG. 3. Examples of typical oocytes in vitro matured after culture in conditions consisting of NCSU23 medium with 1.5% FSH, 7.5% serum, and three follicles per well. A) Mature oocyte with first polar body (bar = 25 μm). B) Chromatin configuration immature oocyte: top, chromosomes (arrow); bottom, first polar body (arrow). Original magnification ×1000. C) Spindle configuration in mature oocyte. Original magnification ×1000.

of FSH, serum, and number of follicles per well on follicle growth patterns in vitro. Moreover, the oocyte from the preantral follicle maturation, fertilization, and embryonic development confirmed the orthogonally designed screening trial.

In this culture system, Figure 1C shows that three follicles and five follicles per well induced similar growth rates and larger than was induced with one follicle per well during the first day of culture. Afterward, however, three follicles (the absence of direct contact) per well produced the best growth. The results suggest an optimal number of follicles per well may support one another by interfollricular paracrine regulation (such as growth factor secretion). Conversely, an excess number of follicles per well may inhibit growth by attaching to one another, and then forming a single mass. Spears et al. [35] reported that dominant follicles can suppress the growth of neighboring follicles with which they come in contact. This is in agreement with our observation that five follicles per well restrained all follicles by direct contact among follicles. However, our data showed that in the absence of contact, three follicles per well can support each another.

Homologous serum has previously been shown to be essential to the maintenance of normal intact follicle organization [10]. Wu et al. [20] have shown that there is a higher requirement for serum factors in primary stage mouse follicles than during later development. Cecconi [23] used 10% serum for sheep preantral follicle development in vitro. In the present study, a slightly increased serum concentration and number of follicles per well induced a rapid growth and high proportion of antrum formation. This indicates that the presence of a higher serum concentration may improve nutrient supply and gas exchange in domestic animal follicles. However, Newton et al. [24] reported that preantral follicles were cultured successfully to the antral stage in serum-free medium.

Figure 1B shows an increasing mean growth rate and antrum formation with increasing concentration of FSH to a maximum of 1.5 ng/ml. The absence of FSH produced slow follicular growth and no antrum formation. The results demonstrated that porcine preantral follicles can be efficiently grown in vitro under the effect of FSH, and that an optimal concentration of FSH is important for antral development in the present culture system. This is consistent with the previous findings that FSH was required for the growth of preantral follicles [7, 10, 36–40]. FSH receptors are expressed on granulosa cells of preantral and antral follicles, and the theca layer [41, 42]. Previous studies suggest that FSH acts on these cells and stimulates the expression of growth factors (such as kit-ligand, keratinocyte growth factor, hepatocyte growth factor, and transforming growth factor) [43]. Moreover, FSH serves as the primary inducer of granulosa cell maturation. FSH also activates the proliferation of granulosa cells, enhances aromataseenzyme activity, and promotes the expression of LHR [44–46]. However, in other culture environments, FSH may not be required elements for in vitro follicular development. Gonadotropins did not seem to be essential in the long-term cultures of the cumulus-oocyte complexes from early follicle stages described by Eppig and Schroeder [7] and Daniel et al. [47]. Moreover, In sheep, 7% of preantral follicles reached the antral stage of development in the absence of gonadotropin [24]. Antra-like cavity formation was observed in some human preantral follicles from follicular aspirates in culture without gonadotropin [22]. One explanation for these findings is that the oocyte-granulosa cell complexes or follicles received FSH stimulation in vivo before isolation.

In conclusion, the present study has shown that specific combinations of factors at appropriate doses are required to induce rapid growth of preantral follicles and antrum formation in vitro and, thereafter, oocyte maturation, fertilization, and embryonic development. We have provided the first evidence that oocytes from preantral follicles of a large animal species can undergo fertilization and develop to the blastocyst stage under a suitable culture system.

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