Effects of varying gonadotrophin dose and timing on antrum formation and ovulation efficiency of mouse follicles \textit{in vitro}

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\textbf{BACKGROUND:} This study tested factors affecting mouse follicle growth \textit{in vitro}, to determine end-points marking follicle function \textit{in vitro}. \textbf{METHODS:} Pre-antral follicles (mean 137 \(\mu\)m) from B6CBF1 mice were cultured in a substrate-adherent system for \(\leq 14\) days. FSH (0–1000 mIU/ml), day of HCG (1.5 IU/ml, days 9–14) and protein supplement [fetal calf serum (FCS) \((\times 2)\), mouse serum \((\times 2)\), hypogonadal (hpg) mouse serum or human serum albumin (HSA)] were varied. Follicle survival, timing of antrum formation, incidence of ovulation within 16, 24, 40 and 48 h of HCG, and oocyte growth were assessed. \textbf{RESULTS:} FSH (100 mIU/ml) produced the best antral development \((P < 0.001\) versus 10 and 1000 mIU/ml). Antra were observed from day 5. Transient antra formed occasionally in the absence of FSH. By 14 days, significant senescence had occurred \((P < 0.001)\), but the proportion of follicles ovulating within 16 h of HCG declined from day 9 onwards, indicating this to be a more sensitive marker of follicle responsiveness. Optimal growth occurred in 5% FCS \((\times 2)\) or hpg mouse serum, although fewer follicles ovulated in hpg serum \((P < 0.05)\). No normal growth occurred in normal mouse serum \((\times 2)\) or HSA. Oocytes grew to full size within 9 days with 100 mIU/ml FSH and FCS. \textbf{CONCLUSIONS:} These data provide sensitive end-points for assessing follicle growth \textit{in vitro}.

\textit{Key words:} antrum formation/follicle growth \textit{in vitro}/FSH/in-vitro culture/mouse follicles

\textbf{Introduction}

Mouse pre-antral follicles can be cultured to maturity \textit{in vitro}, permitting the study of follicle growth and ovulation in the absence of systemic influences, such as nervous innervation and blood supply. To date, the optimal procedures have not been determined in depth, although many different approaches and culture techniques have been applied to study a variety of aspects of follicular function in various species. Some culture systems maintain the three-dimensional structure of follicles, singly or in groups (Qvist \textit{et al}., 1990; Spears \textit{et al}., 1996), either in gels (Torrance \textit{et al}., 1989; Carroll \textit{et al}., 1991) or non-adherent conditions (Nayudu and Osborn, 1992; Boland and Gosden, 1994; Hartshorne \textit{et al}., 1994; Spears \textit{et al}., 1994) whereas others allow adherence to the underlying substrate causing collapse of the follicle. Surprisingly, such follicles, which have lost their physiological form and are isolated from systemic influences, remain capable of organizing an antrum and ovulating in response to appropriate culture supplements (Gore-Langton and Daniel, 1990; Cain \textit{et al}., 1995) and can result in fertile oocytes (Daniel \textit{et al}., 1989; Cortvrindt \textit{et al}., 1996). Such a two-dimensional system may have a number of advantages, allowing improved oxygenation, nutrition and access to culture supplements (Cortvrindt \textit{et al}., 1996). Nevertheless, limited information is available on the normality of follicle development \textit{in vitro} under such non-physiological conditions.

We have chosen to explore the gonadotrophin responsiveness of mouse follicles in the substrate-adherent system in order to gain information about follicle longevity and ovulatory efficiency \textit{in vitro}. Such information may be of value in future for the culture of follicles from larger species, such as humans, where growth of intact follicles to full size (>20 mm diameter) is unlikely to be feasible \textit{in vitro}.

\textbf{Materials and methods}

Reagents were obtained from Sigma Chemical Co. (Poole, UK) unless otherwise indicated. Mice (B6CBF1) were housed and bred under government licence at the University of Warwick. Food and water were available \textit{ad libitum}, the light:dark cycle was 12:12 h and all animals were monitored daily. Female mice, aged 3–4 weeks, were humanely killed and the ovaries removed and placed into Leibovitz medium (Gibco, Paisley, UK) containing 0.37% w/v bovine serum albumin (BSA), penicillin (50 mIU/ml) and streptomycin (50 \(\mu\)g/ml). Using fine, syringe-mounted needles (29 gauge, 0.33 mm; Becton Dickinson, Oxford, UK), pre-antral follicles were identified and carefully dissected away from the surrounding tissue. Follicles used for experiments were spherical, the basement membrane intact with the oocyte clearly visible and centrally located, the follicular cells were not degenerate in appearance, and some thecal cells were present. Individual follicles were rinsed with culture medium and placed into 10 \(\mu\)l drops of culture medium under pre-equilibrated mineral oil in 60×60 mm uncoated tissue culture grade Petri dishes (Nunc; Gibco) using a system similar to one previously described (Cortvrindt \textit{et al}., 1996). Two days later, addition of 10 \(\mu\)l of fresh
medium brought the volume to 20 µl per drop. Thereafter, every second day, 10 µl of medium was removed from each drop and 10 µl of fresh medium was added. Cultures were incubated at 37°C in 5% CO₂ in air.

The culture medium comprised minimal essential medium alpha (MEMα; Gibco) containing 5% heat-inactivated fetal calf serum (FCS; Gibco) (or alternative sera as indicated below), penicillin (50 µg/ml), streptomycin (50 µg/ml) and apo-transferrin (10 µg/ml). FSH (HP Metrodin; Serono, Welwyn Garden City, UK) was prepared in unsupplemented culture medium and stored in aliquots at −20°C until used to produce final concentrations of 0–1000 mIU/ml.

Cultured follicles were observed daily to assess cellular attachment, growth, formation of antrum-like cavities, premature extrusion of oocyte and degeneration. Dimensions were measured only for follicles in which the basement membrane was intact and measurements were made of the area inside the basement membrane. Two perpendicular directions were measured using a previously calibrated eyepiece graticule on a dissecting microscope at a magnification of at least ×800 and the mean was calculated and used for analyses. Once the basement membrane had ruptured, follicle appearance was described qualitatively. Follicles were defined as surviving when the oocyte was retained, appearing round in shape and follicle cells were not degenerate. HCG (Pregnyl; Organon, Oss, The Netherlands) was added to surviving follicles at a final concentration of 1.5 IU/ml and ovulation was noted 16, 24, 40 and 48 h later. Ovulation was defined as the complete release of the oocyte from the follicle after HCG addition. The presence or absence of attached cumulus cells was noted.

To assess viability, live and dead cells in some cultured follicles were detected by staining with carboxy fluorescein diacetate (CFDA; Sigma, Poole, UK) and propidium iodide (PI; Sigma, Poole, UK) respectively using a modification of a published method (Garner et al., 1986). CFDA (Sigma) was prepared as a stock solution of 0.46 mg/ml in phosphate-buffered saline (PBS), stored in aliquots at −20°C wrapped in foil and was used at a 1:50 dilution to give a final concentration of 9.2 µg/ml. PI was prepared as a stock solution of 1 mg/ml in PBS, stored at 4°C and was used at a 1:25 dilution to give a final concentration of 40 µg/ml. Follicles in situ in culture were washed in serum-free culture medium (MEMα) and medium containing CFDA was added. This was incubated at 37°C for 20–30 min and then the solution was carefully removed. The follicle was washed with serum-free culture medium (MEMα) and then PI-containing medium was added. After 5 min at room temperature, PI was removed and replaced with serum-free culture medium (MEMα). The follicles were viewed using an inverted fluorescence microscope (Nikon) equipped with rhodamine, fluorescein isothiocyanate and triple filters (to observe staining with PI, CFDA and both respectively).

The following experiments were undertaken:

**Log dose–response curve to FSH (n = 322 follicles)**

Mouse follicles were cultured in the presence of 0, 10, 100 and 1000 mIU/ml FSH. The day upon which antrum formation was first observed was noted. HCG was added on day 12 and ovulation noted at 16, 24, 40 and 48 h post HCG addition.

**Effect of protein source (n=180 follicles)**

Follicles were cultured in the presence of six different protein sources for 12 days in the presence of 100 mIU/ml FSH. HCG was added on day 12 and ovulation noted at 16, 24, 40 and 48 h post HCG addition. The protein sources used were as follows: human serum albumin (HSA, 1%; Immuno Ag, Vienna, Austria); mouse serum (5%, two different batches, MS2 and MS7); FCS (5%, Gibco); embryo stem cell tested FCS (ESFCS, 5%; Gibco); hypogonadal mouse serum (hpgMS, 5%, a kind gift from Prof. H.Charlton, Oxford). Sera not already treated were heat-inactivated by incubation at 56°C for 40 min.

**Day of HCG addition (n=651 follicles)**

Follicles were cultured in the presence of 100 mIU/ml FSH and 5% FCS. HCG was added on day 9, 10, 11, 12 or 14. Ovulation was noted at 16, 24, 40 and 48 h post HCG.

**Oocyte growth (n=200 follicles)**

Oocyte diameter was measured throughout culture to ovulation (HCG day 10) without disruption of the follicles. These measurements were approximate as it was not possible to distinguish the exact boundary of the oocyte through the surrounding cumulus cells. Measurements were the average of two perpendicular directions. Oocyte diameters were also measured for follicles with or without HCG addition on days 9, 10, 11 and 12. These measurements were made using an inverted microscope, after stripping off any cumulus cells, and included the zona pellucida.

For follicle survival and ovulation, 2×2 contingency tables with χ²-test were performed. Where a mean and SD are quoted (such as for follicle and oocyte diameter), Student’s t-tests were performed for normally distributed data. Unless otherwise stated, data are expressed as mean ± SD and P < 0.05 was considered significant.

**Results**

In this culture system, when appropriate conditions were applied, follicle development occurred with a high level of success. Pre-antral follicles for culture had diameters on day 0 of 138.3 ± 18.0 µm (range 95–205). The cultured follicles initially became attached to the culture dish via theca cells, with granulosa cell proliferation causing rupture of the basement membrane and subsequent migration over the thecal cells. Antrum-like cavities were formed and a phenomenon analogous to ovulation occurred in response to HCG. Figure 1 summarizes the stages of follicle growth in vitro.

When an ovariolytic stimulus (HCG, 1.5 IU/ml) was added to surviving follicles, the oocyte was often released into the medium, usually surrounded by muficid cumulus cells (Figure 1i). However, the percentage of oocytes with attached cumulus cells decreased as the time from HCG to ovulation increased, as shown in Figure 2.

**FSH concentration**

The follicles cultured in 0, 10, 100 and 1000 mIU/ml FSH had diameters of 139.1 ± 1.7, 139.4 ± 2.3, 135 ± 1.8 and 140 ± 2.3 µm (mean ± SEM) respectively at the start of culture (not significant). The results of varying FSH concentration upon follicle survival and ovulation in response to an HCG stimulus are summarized in Figure 3 and the development of the follicles in vitro up to the time of HCG addition is presented in Table I. The majority of the follicles (57%) cultured without FSH prematurely released their oocyte between day 4 and day 12. Other follicles appeared degenerate (17%), did not grow (5%) or follicular cells were sparsely attached to the bottom of the dish (6%, Figure 4a in comparison with c). Some follicles initially appeared healthy but then degenerated (5%) with only 10% surviving to HCG.

When follicles were cultured in 1000 mIU/ml of FSH, 75% survived but the total and timely ovulation rates were significantly (P < 0.001) lower than with 100 mIU/ml FSH, i.e. a high...
FSH dose and mouse follicle growth in vitro

Figure 1. Mouse pre-antral follicles cultured to ovulation in the substrate-adherent system. (a) Day 1. Follicle of diameter 158 µm. Oocyte is centrally located with approximately three layers of granulosa cells. The basement membrane is intact with thecal cells attached. (b) Day 2. Follicle of diameter 221 µm. Thecal cells have attached to the bottom of the dish but the basement membrane is still intact. (c) Day 3. Granulosa cells have broken through the basement membrane in some follicles and attached to the thecal cells. (d) Day 4. The attached granulosa cells proliferate over the thecal cells and the bottom of the culture dish. The oocyte remains centrally located. (e) Day 5. Some follicles have started to form small antral-like cavities within the granulosa cells. (f) Day 6. Antral cavities persist and granulosa cells continue to proliferate. (g) Day 8. The antrum enlarges further, with the oocyte in its cumulus mass clearly visible in the centre of the cavity. (h) Day 10. The oocyte is in the centre of a large antrum, attached to the follicle by ‘stalks’ of granulosa cells and surrounded by non-mucified cumulus cells. (i) Day 11. Within 24 h of HCG addition, the oocyte (top left) had been released by this antral follicle into the surrounding medium with mucified cumulus cells attached. Original magnifications: (a) ×200, (b–i) ×100. All scale bars = 100 µm.

Figure 2. Percentage of ovulated oocytes having attached cumulus cells at 16, 24, 40 and 48 h post HCG addition on day 10. The total number of follicles used was 1241, which was a composite of all follicles cultured under standard conditions (fetal calf serum, 100 mIU/ml FSH) and having HCG on day 10. This includes control groups from experiments not reported in this paper, e.g. examining the effects of other substances in vitro, so the numbers do not tally with those reported in Materials and methods. The numbers ovulating at each time point are: <16 h, 1067; 16–24 h, 12; 24–40 h, 158; 40–48 h, 4. Of these, 1045/1067, 10/12, 9/158 and 0/4 had cumulus cells attached.

The percentage of follicles (39% of all follicles cultured) did not ovulate in response to HCG. The visual assessment of follicle viability was confirmed using vital stains. Follicles cultured without FSH contained many PI-stained cells and those with 100 mIU/ml FSH contained mainly CFDA-stained cells. The proportions of live and dead cells were analysed qualitatively. Representative examples are shown in Figure 4c and d.

Follicular antrum formation was significantly ($P < 0.001$) impaired when follicles were cultured with 0, 10 or 1000 mIU/ml FSH compared with 100 mIU/ml FSH (Figure 5). The antral cavities that were formed in the absence of FSH had collapsed by the end of culture, suggesting that the follicles could not sustain antral development without FSH. The starting diameter of follicles that formed antra without FSH present was 151.6 ± 8.8 µm, which is greater than the mean diameter of follicles cultured without FSH. It is also greater than the mean diameter (136.5 ± 17.2 µm) of follicles cultured with 100 mIU/ml FSH which formed antra in vitro.

Figure 3. Effect of FSH concentration on follicle survival and time of ovulation. Ovulation was significantly more likely with 100 mIU/ml FSH ($P < 0.001$). Number of follicles = 116, 55, 95, 56 at 0, 10, 100 and 1000 mIU/ml FSH respectively.
Figure 4. Follicles cultured with and without FSH (original magnification ×100, scale bars = 100 μm). (a) Light micrograph of follicle without FSH on day 8 of culture. Follicular cells are sparsely attached and dark in appearance with some rounded up on the bottom of the dish. No antral cavities were formed in this follicle and by day 9 the oocyte had been prematurely released. (b) Light micrograph of follicle cultured with 100 mIU/ml FSH on day 8 of culture. Follicular cells are attached to the dish and have proliferated. A large antrum has formed with the oocyte surrounded by cumulus in the centre of the cavity. (c) Fluorescence micrograph of a day 8 follicle cultured without FSH, stained with the vital stains carboxy fluorescein diacetate (CFDA) and propidium iodide (PI). The red fluorescence and lack of green indicates non-viable cells. (d) Fluorescence micrograph of a day 8 follicle cultured with 100 mIU/ml FSH, stained with CFDA and PI. The majority of cells have stained green, including the oocyte, indicating their viability.

Table 1. Follicle development in vitro to day 12 (day of HCG addition) in the presence of various concentrations of FSH

<table>
<thead>
<tr>
<th>FSH concentration (mIU/ml)</th>
<th>Survival to HCG (%)</th>
<th>Premature release of oocyte (%)</th>
<th>Degeneration (%)</th>
<th>No growth (%)</th>
<th>Sparse attachment of follicular cells (%)</th>
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<tr>
<td>0</td>
<td>10</td>
<td>57</td>
<td>22</td>
<td>5</td>
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<td>1000</td>
<td>75</td>
<td>16</td>
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<td>9</td>
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A concentration of 100 mIU/ml FSH was used for subsequent experiments.

Effect of protein source

All follicles cultured in HSA or either batch of normal mouse serum degenerated by day 9. In these conditions, follicular cells did not attach to the culture dish, substantial follicle growth did not occur and antral cavities were not formed. Nearly half the follicles cultured with MS2 (43%) and MS7 (40%) prematurely released their oocyte, compared with 20% in HSA, 10% in hpgMS, 7% in ESFCS and 3% in FCS.

In hpgMS, ESFCS and FCS, follicular cells attached to the culture dish, with follicles developing antra and ovulating in response to HCG. The follicles appeared visibly similar during culture and the survival rates were not significantly different in the different sera (87–93%, NSD); however, the ovulation rates varied, as shown in Figure 6. A significantly lower proportion of follicles cultured with hpgMS was able to respond to the ovulatory stimulus than in ESFCS (P < 0.05)

Day of HCG addition

When a fixed concentration of HCG was added to surviving follicles on days 9, 10, 11, 12 or 14, variations were noted in the proportions ovulating within 16, 24, 40 and 48 h later.

A total of 651 follicles of initial diameter 139.0 ± 18.5 μm on day 0 was cultured. The survival rate was significantly (P < 0.001) lower on day 14 (74%) than on days 9–12 (87–95%) as shown in Figure 7. The proportion of follicles ovulating also varied with the duration of culture. Follicles with HCG added on day 11 or later had a significantly (P < 0.01–0.001) reduced proportion of follicles with timely ovulation (within 24 h of HCG) compared with days 9 or 10. Few follicles that ovulated late (>24 h after HCG) had cumulus cells attached, irrespective of the day of HCG addition.
FSH dose and mouse follicle growth *in vitro*

**Figure 5.** Effects of FSH concentration on antral cavity formation (total number of follicles = 266). (a) Antrum formation during culture with 0, 10, 100 and 1000 mIU/ml FSH, using 60, 55, 95, 56 follicles respectively. Antrum formation was significantly more likely with 100 mIU/ml FSH than at the other concentrations tested. (*P* < 0.001). (b) Effects of FSH on the timing of antrum formation *in vitro*. The figure is a cumulative chart showing the days on which follicles exposed to different concentrations of FSH first formed antra. Some follicles formed antra on days 7 and 8 when cultured with 0 mIU/ml FSH, which then collapsed after a maximum of 2 days. The single follicle that formed an antrum when cultured with 10 mIU/ml formed the cavity late (on day 12). Follicles cultured with 100 mIU/ml started to form antra by day 5, with most on day 8 or 9. Follicles cultured with the highest amount of FSH (1000 mIU/ml) formed antra occasionally throughout the culture, the earliest observed on day 7.

However, 92% of follicles that ovulated within 24 h of the HCG stimulus had mucified cumulus cells attached. Cumulus cell attachment depended on the day of HCG addition. A significantly (*P* < 0.001) higher number of follicles had mucified cumulus cells attached to the oocytes when ovulating within 24 h of HCG addition on days 9 (99%), 10 (97%), 11 (95%) and 12 (86%), compared with day 14 (36%). A proportion of follicles (ranging from 19% to 32%) did not ovulate when HCG was added on day 9–14.

**Oocyte growth during culture**

A gradual increase in size of all measured oocytes was observed (Figure 8). The mean oocyte diameter was 53.8 ± 8.8 µm at the start of culture (day 0) with a mean follicle diameter of 132.8 ± 12.5 µm. The oocyte diameter gradually increased throughout the culture period to reach a mean diameter of 76.3 ± 3.9 µm by day 9, 75.7 ± 4.1 µm by day 10 and 76.3 ± 3.9 µm on day 11, after HCG addition on day 10 (Figure 8). A separate series of experiments showed that oocyte diameter was not affected by the addition of HCG on days 9, 10, 11 and 12 (Table II).

**Figure 6.** Relative efficiency of three sera supporting follicle survival on ovulation *in vitro*. There were no significant differences in survival and timely ovulation rates; however, significantly fewer follicles cultured with hpgMS were able to respond to the ovulatory stimulus, in comparison with those in ESFCS (*P* < 0.05). The number of follicles at each time point was 30. hpgMS = hypogonadal mouse serum; ESFCS = embryo stem cell tested fetal calf serum; FCS = fetal calf serum.

**Figure 7.** Effect of day of HCG addition on the timing and efficiency of ovulation *in vitro*. HCG was added to 94 follicles on day 9, and to 181, 97, 183 and 96 follicles on days 10, 11, 12 and 14 respectively.
Table II. Oocyte diameter on different days of culture in the presence and absence of HCG

<table>
<thead>
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<th>Day 9</th>
<th>Day 10</th>
<th>Day 11</th>
<th>Day 12</th>
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<tr>
<td></td>
<td>−HCG</td>
<td>+HCG</td>
<td>−HCG</td>
<td>+HCG</td>
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<tr>
<td>No. of follicles</td>
<td>23</td>
<td>26</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>Oocyte diameter (µm)</td>
<td>73.3 ± 3.9</td>
<td>72.2 ± 3.7</td>
<td>73.0 ± 2.4</td>
<td>71.0 ± 3.3</td>
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Data are mean ± SD.

Figure 8. Growth of oocytes during follicle culture (n = 12 at all points).

Discussion

In this series of experiments, we have characterized the response of mouse follicles in culture to a variety of different conditions in terms of their survival, antrum formation, ovulation efficiency and ovulation timing. Growth of pre-ovulatory follicles from a pre-antral population averaging ~138 µm diameter took ~10–12 days, as compared with ~12–14 days (Cortvrindt et al., 1996). It seems that development in vitro may be accelerated compared with that in vivo, where small pre-antral follicles take ~16 days to become pre-ovulatory (Pederson, 1970). Follicles in culture are free from systemic contacts and many intra-ovarian influences. This may possibly decrease exposure to inhibitory feedback from other growing follicles (Spears et al., 1996) and could partially explain the relatively high numbers of individually cultured follicles which can rapidly attain maturity in vitro.

Our culture system is based upon an established method (Cortvrindt et al., 1996), but there are several notable differences in our approach. The mice used here were 28 days old in comparison with 14 days. We selected older mice, approaching puberty to reduce the potential confounding influence of sexual immaturity which can have a variety of effects on oocyte and follicle function (Bao et al., 2000; Ledda et al., 2001). We omitted insulin from our cultures since our preliminary experiments showed it to have no beneficial effect, and others have demonstrated interactions between insulin and FSH to the detriment of mouse follicle and oocyte development in vitro (Latham et al., 1999). We used a highly purified urinary preparation of FSH instead of recombinant FSH because of uncertainty about the physiology of different isoforms in the artificial preparation (Ydning Andersen et al., 1999). Urinary human FSH has since been shown to have a slightly higher potency than recombinant human FSH on mouse follicle growth in vitro (Liu et al., 2000a).

In this study, we monitored ovulation at 16, 24, 40 and 48 h after a fixed maximal dose of 1.5 IU/ml HCG. This dose was presumed maximal since 1 IU/ml was found to be a maximal ovulation-inducing stimulus in a non-adherent system for mouse follicle culture (Rose et al., 1999). In vivo, ovulation occurs 12–14 h post HCG (Hogan et al., 1986; Yoshimura and Wallach, 1987). In some cultured follicles, ovulation was delayed until ≥24 h after HCG, possibly indicating suboptimal pre-HCG development. The significance of late ovulation is not known. Since ovulation in vivo occurs promptly, we thought that ovulation within 16 h indicated good HCG responsiveness, and anything later to be potentially indicative of suboptimal follicular development. The proportion of follicles showing this delayed response varies with the culture conditions or is evident from the illustrations (e.g. Figures 3, 6 and 7) in which the timings of ovulation are indicated. Many late ovulated oocytes had reduced or no cumulus surrounding, indicative of a loss of follicle/oocyte communication and potentially associated with suboptimal growth or post-maturity. Our data showed that prolonged culture beyond 12 days was associated with a significantly lower survival rate; however, the timely ovulation rate had begun to decline even from day 9 (Figure 7), suggesting that ovulation timing is a more effective indicator of follicle viability than survival in culture per se. Cortvrindt et al. (1996) assessed the response to a dose of HCG combined with epidermal growth factor (EGF) over a similar period of time in terms of cumulus cell mucification. They concluded that after day 12 (days 14 and 16) there was a significant reduction in the ability of cumulus cells to mucify. This may have related to the onset of spontaneous luteinization on day 14, although antral development continued and germinal vesicle arrest was maintained in oocytes until day 16. However, when oocyte maturation was used as the end-point, they determined days 12 and 14 to be optimal for the HCG/EGF stimulus, whereas we have shown that the chances of timely ovulation in response to HCG have already declined substantially by day 12 (Figure 7). EGF is known to improve oocyte maturation and cumulus mucification in response to gonadotrophins in vitro (Boland and Gosden, 1994; Smitz et al., 1998), which may have contributed to this difference. These data show that aspects of granulosa cell function may be compromised by extended in-vitro development before the ovulatory stimulus.

The response to HCG requires the presence on granulosa
cells of LH receptors, which are known to be stimulated by the action of FSH, indicating the importance of appropriate priming. The log dose–response curve, showing the influence of FSH priming upon ovulation in vitro, revealed that high as well as low concentrations of FSH were suboptimal. Our optimal dose of 100 mIU/ml conforms with that found by Nayudu and Osborn (1992) who measured growth in non-attached cultured follicles over a 6 day period. While it is already known that absence of FSH is incompatible with normal follicle growth in vitro (Qvist et al., 1990; Nayudu and Osborn, 1992; Cortvrindt et al., 1996), an upper threshold for optimal FSH priming in the substrate-adherent system has not previously been ascertained. However, it might be expected that excessive exposure to gonadotrophins would result in receptor down-regulation, potentially leading to a suboptimal follicular response (LaPolt et al., 1992).

Antrum formation in vivo begins at a diameter of ~200 µm (Spears et al., 1994). In this culture system, antrum formation was not evident until after the follicles had attached to the substrate and lost their three-dimensional shape, so a direct comparison with in-vivo conditions is not possible. Under suboptimal FSH concentrations, antrum formation in vitro was limited (Figure 5), including when excess FSH was present. Cumulus cell function is known to be compromised by excess FSH causing inappropriate formation of LH receptors (Eppig et al., 1998), however, it is uncertain whether cumulus cells may play a role in the formation of the antrum, as the mechanism remains unclear. Without FSH, only transient antra were observed, persisting for a maximum of 2 days and collapsing by day 9. This probably indicates carryover of FSH effect from the in-vivo situation. Such carryover appears likely since Nayudu and Osborn (1992) found that antrum formation did not occur in the absence of FSH, but when FSH was added to the culture medium during the first 2 days only, the majority of follicles continued to grow and some formed antra.

In our series, when 100 mIU FSH was present, antrum formation was first evident on day 5, which is earlier than its initiation on day 8 observed by Cortvrindt et al. (1996). Also, they did not observe antrum formation in follicles cultured without FSH, in contrast with our data showing that some follicles lacking FSH supplementation formed antra transiently. This difference may relate either to the difference in size range of follicles cultured (85–145 µm compared with 95–205 µm in this study). Alternatively, the potential difference in potency of FSH supplements (Liu et al., 2000a) or their isoform mixtures may have had some effect. It is known that the least acidic FSH isoforms cause accelerated antrum formation in vitro relative to the more acidic forms (Vitt et al., 1998), but their physiological relevance may be limited by their short half-life in vivo (Chappel, 1995) or to a precise time around the late follicular phase (Yding Andersen et al., 1999). Acidic forms are more prevalent in post-menopausal urine, the source of FSH used in this study, than serum (Wide and Wide, 1984); however, urinary preparations and recombinant preparations of FSH appear to contain a similar profile of isoforms by isoelectric focusing (Chappel, 1995). The optimal combination of isoforms for follicle development remains uncertain.

It is customary to include some form of protein supplement in culture media, often serum or a serum-derived protein(s) such as albumin. This is believed to serve several functions: buffering, binding of potentially toxic ions, nutrition, and potentially providing growth factors and other factors supportive of the cultured cells. However, the variability associated with sera especially is undesirable, and less complex alternatives such as albumin or synthetic replacements are sought. Our experiments have shown that supplementation with HSA would not support mouse follicle development in this culture system. This batch was in routine use for human IVF and known to be non-toxic. Most authors using adherent culture of mouse follicles employ FCS (Liu et al., 2000b), although rat follicles have been cultured in a substrate-adherent manner with BSA (Gore-Langton and Daniel, 1990). Intact mouse follicle cultures are usually supplemented with 5% mouse serum (Spears et al., 1996; Rose et al., 1999).

Interestingly, neither of two batches of commercially available normal mouse serum (a mixture of male and female) as used previously for in-vitro follicle culture in a non-attached system (Hartshorne et al., 1994) supported follicle development here. In contrast, hypogonadal mouse serum, which had also been used previously in the spherical culture system (Hartshorne et al., 1993), did support follicle growth in this series in the presence of supplementary FSH. Hypogonadal mice lack the GnRH gene and consequently have extremely low levels of FSH and LH. Their gonads remain permanently infantile unless exogenous FSH is administered (Mason et al., 1986). This difference in follicle development in vitro between normal and hpg mouse serum, both supplemented with FSH in vitro, is interesting. It could relate to the levels of various other factors in the sera (for example steroids, other gonadotrophins such as LH, or binding proteins), or to the direct access to granulosa cells afforded by substrate-adherent follicles. Further investigation is needed to elucidate the key components causing the difference. While there was no difference in timely ovulation between hpg mouse serum and the two sources of FCS, more follicles remained unresponsive to the ovulatory dose of HCG, which may indicate that some factor is lacking in hpg serum which affects their priming. For example, androgens have positive effects upon intact follicle growth in vitro (Murray et al., 1998).

Oocytes in cultured follicles grew from an initial diameter of 54 µm to a maximum diameter of 76 µm by day 9. The oocytes had already entered the growth phase before culture began, and as expected, follicle development continued after oocytes were fully grown. These results are slightly different from those of Cortvrindt et al. (1996) who found a shallower growth curve which continued throughout the full 16 days of their culture (Cortvrindt et al., 1996), although their starting and fully grown oocyte diameters were similar to ours. These final oocyte diameters approximate to those occurring in vivo, in contrast to other systems where the final diameter of cultured oocytes has been reported as smaller than normal (Eppig and Schroeder, 1989; Eppig and O’Brien, 1996). Developmental competence is not achieved until oocyte size exceeds a threshold of ~80% (Bar Ami and Tsasfriri, 1981) so size is an important measure of oocyte function. The differences in rates of growth could possibly relate to the different ages of the mice (Bao
et al., 2000). The exposure of fully grown oocytes to HCG did not affect their diameters. Oocyte growth requires gap junctional communication with granulosa cells to be maintained during follicle development. The production of fully grown oocytes is therefore an indication of the ongoing intercellular connections in a coordinated manner within the follicle structure, despite its attachment to the underlying substrate.

In conclusion, we have demonstrated the ability of mouse follicles to grow, form antrum, and ovulate in vitro in an adherent culture system. The efficiency of these key markers of follicle function may be affected by the starting material and by the gonadotropic support and culture conditions to which it is exposed. In our experiments, optimal results in terms of survival, follicular appearance and antrum formation were obtained with MEMt supplemented with 5% heat-inactivated FCS and 100 mIU/ml FSH. This protocol resulted in survival of 87% of follicles to day 12. Addition of 1.5 IU/ml HCG on day 9 resulted in the greatest proportion (71%) of follicles ovulating within 16 h, indicating a prompt response to HCG, although statistically similar results were obtained for HCG addition on day 9.

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Boland, N.I. and Gosden, R.G. (1994) Effects of epidermal growth factor on survival, follicular appearance and antrum formation were obtained with MEMt supplemented with 5% heat-inactivated FCS and 100 mIU/ml FSH. This protocol resulted in survival of 87% of follicles to day 12. Addition of 1.5 IU/ml HCG on day 9 resulted in the greatest proportion (71%) of follicles ovulating within 16 h, indicating a prompt response to HCG, although statistically similar results were obtained for HCG addition on day 9.


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