Few instead of many: human follicle collection from follicular aspirates at oocyte retrieval

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BACKGROUND: The aim of this study was to determine if follicular aspirates obtained during oocyte retrieval for IVF were a good source of ovarian follicles for research purposes. METHODS: Follicular aspirates from 86 patients were collected and examined for the presence of follicles, and histological examination of tissue sample found was undertaken. RESULTS: Follicles were only obtained from aspirates of seven out of a total of 86 patients. From these samples a total of 14 follicles was found. The follicles were primordial, primary or secondary, 40–80 µm in diameter. Three of these recovered follicles were cultured and all degenerated within 2 days. In all aspirates some groups of granulosa cells that did not contain follicles or oocytes were found, as was vaginal epithelium that was also identified and verified by histology. CONCLUSIONS: Follicular aspirates are not a useful source of human follicles. Some structures found in the aspirates may be erroneously identified as follicles.

Key words: follicular fluid/human/IVF/oocyte/ovarian follicle

Introduction

In humans, the availability of competent mature oocytes from immature follicles would greatly improve the prospects for treatment of infertile couples, both reducing the burden of intensive drug stimulation of the ovaries, and potentially enhancing the pregnancy rate, with the added benefit of providing readily available oocytes for donation and research (Hartshorne, 1997). In-vitro growth of immature follicles is a technique that could have applications in human IVF. Methods for growing immature follicles have been developed for rodents (Eppig and O'Brien, 1996) and a range of domestic species (Telfer et al., 2000). These techniques have not been developed in humans because of the difficulty in obtaining immature follicles. A recent publication suggested that follicular aspirates obtained from oocyte retrieval are a good source of human follicles and oocytes (Wu et al., 1998). This study suggested that around 51 follicles per patient could be obtained from this source and that some of them could be cultured for up to 28 days to obtain mature oocytes (Wu et al., 1998). This new source of human follicles and oocytes has been regarded as having obvious advantages for research and clinical practice (Edwards and Beard, 1998).

The aim of this study was to determine whether immature follicles could be obtained from follicular aspirates obtained in our IVF programme, and if these follicles could survive in vitro.

Materials and methods

Follicular aspirates were obtained from 86 patients undergoing oocyte retrieval for IVF. After gonadal suppression by GnRH agonists (Buserelin, Suprecur; Hoechst, Frankfurt-am-Main, Germany) or an antagonist (Cetrotide; Asta Medica, Dresden, Germany), controlled ovarian hyperstimulation was achieved by recombinant FSH (Gonal F; Serono Nordic, Stockholm, Sweden) or Puregon (Organon, Oss, The Netherlands). Ovulation was induced by hCG (Profasi; Serono or Pregnyl; Organon) when the largest follicles had reached a diameter of at least 18 mm. Follicles were aspirated transvaginally 36 h later with ultrasound guidance using a single-lumen needle. The age (mean ± SD) of patients was 33.1 ± 3.7 years (range 22–39). The volume (mean ± SD) of the pooled follicular aspirates from each patient was 27.2 ± 17.9 ml (range 10–90). Detailed information on the patients is given in Table I.

The ethics committee of Karolinska Institutet, Huddinge University Hospital approved the study.

Identification of follicles

The first aspirate from each ovary was collected in order to obtain follicles from the ovarian cortex caught by the aspiration needle when the ovary was punctured initially. Aspirates with high concentrations of blood were avoided. After the oocyte–corona–cumulus complexes were removed as part of the clinical treatment of the patient, the remainder of the follicular aspirates was collected into sterilized centrifuge tubes and centrifuged at 200 g for 10 min. The supernatants were removed.

For the first 41 patients, the pellets were resuspended and examined directly. For the following 45 patients, steps were taken to remove erythrocytes as follows: 27 ml of distilled water was added to each tube, which was shaken vigorously and left for 20–30 s. Then 3 ml of 10×PBS (phosphate-buffered saline) were added to each tube to restore normal osmolarity. After centrifugation at 200 g for 3 min,
The supernatants were removed. The pellets were resuspended and examined. All the pellets were dissolved in 0.5 ml pre-equilibrated α-MEM (Invitrogen Inc., Scotland, UK) and examined both under a dissection microscope at ×20–70 magnification and under an inverted microscope at ×100–400 magnification.

In most cases, it was easy to distinguish the follicles from other structures according to the size, shape, brightness and arrangement of the cells. When any doubt existed, the distinguishing procedures were performed.

Distinguishing between follicles and granulosa cell masses

Under the inverted/dissection microscope, some granulosa cell masses resembled follicles. Two methods were used to check whether these cell aggregates contained oocytes. Firstly, granulosa cell aggregates were cultured on 96-well plates (Nunclon; Nunc, Denmark) individually. They were primordial, primary or secondary. Their diameters being 40–80 μm (Figure 1).

The second method involved removing the granulosa cells enzymatically and mechanically. The granulosa cell masses were incubated in 80 IU/ml hyaluronidase (HYASE-10°/H11003; Vitrolife, Göteborg, Sweden) for 20 s. After incubation, the granulosa cells were separated using 27 G needles.

Culture of immature follicles

Three follicles (primordial and/or primary) from two patients were cultured individually in the same way as for the granulosa cells.

Histology examination

A small piece of tissue collected from the aspirates was fixed in Bouin’s fixative. After fixation, the tissue was dehydrated and embedded in paraffin. The tissue was cut into serial sections of 4 μm and stained with haematoxylin and eosin (H&E) and then examined under light microscope.

Statistical analysis

The χ²-test was utilized for analysis of differences in the fertilization rate, pregnancy rate and percentage of mature oocytes between two groups. Fisher’s exact test was used when the value was ≤5. Student’s t-test was used to compare age differences in the patients and the number of the oocytes retrieved between two groups. P < 0.05 was considered significant.

Results

In 79 patients no ovarian follicles were identified from the aspirates. Fourteen follicles were obtained from seven patients, one to five follicles per patient. All these follicles were individual. They were primordial, primary or secondary, their diameters being 40–80 μm (Figure 1).

There were no significant differences between the patients with or without recovered immature follicles with respect to their age, number of the oocytes retrieved, fertilization rate and pregnancy rate. Patients with recovered immature follicles had a lower proportion of mature oocytes than those without (75.7 versus 90.3%, P < 0.001).

Granulosa cell masses that resembled follicles were cultured and they tended to adhere to the bottom of the plate to form a single layer instead of round aggregates during culturing. They degenerated gradually. The number of granulosa cells decreased and the number of vacuoles increased in the culture (Figure 2). After 4 days culture, the granulosa cells had degenerated completely. No oocytes were visible within these cell aggregates over the culture time.

After incubation in hyaluronidase solution, the granulosa cells were easy to separate. In each cell mass, almost all the granulosa cells were isolated to form a single layer. No oocytes were found in these masses.

All three follicles cultured (from two patients) degenerated within 2 days.

A small thin piece of tissue was found that contained vaginal epithelium resembling cluster of follicles under the inverted microscope. After fixation and routine H&E stain, these ‘foillcles’ were clearly vaginal epithelium as revealed by light microscope (Figure 3).

Table I. Data on patients with (group1) or without (group 2) recovered immature follicles

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (n = 7)</th>
<th>Group 2 (n = 79)</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td>Age (years, mean ± SD)</td>
<td>32.7 ± 4.1</td>
<td>33.1 ± 3.7</td>
<td>NS</td>
</tr>
<tr>
<td>Total oocytes retrieved (mean ± SD)</td>
<td>70 (10 ± 4.5)</td>
<td>930 (11.8 ± 7.0)</td>
<td>NS</td>
</tr>
<tr>
<td>MII oocytes (n, %)</td>
<td>53 (75.7)</td>
<td>840 (90.3)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Immature oocytes (n, %)</td>
<td>17 (24.3)</td>
<td>90 (9.7)</td>
<td>–</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>62.3</td>
<td>67.5</td>
<td>NS</td>
</tr>
<tr>
<td>Pregnancy rate (%)</td>
<td>14.3</td>
<td>28.2</td>
<td>NS</td>
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NS = not significant.
oocyte retrieval required repeated punctures of the ovary, and

Theoretically, it would be possible to obtain many follicles if the needle is seldom pushed through the ovarian surface more than once, making it likely that few follicles will be found. Theoretically, it would be possible to obtain many follicles if oocyte retrieval required repeated punctures of the ovary, and

this may be a possible explanation as to why we did not find as many follicles as Wu et al. reported (Wu et al., 1998). There may be differences in the oocyte retrieval procedures between different IVF units.

We found that some components in follicular aspirates appeared to be follicle-like structures when examined under an inverted microscope. Further analysis revealed these structures to be predominantly granulosa cell aggregates. Vaginal epithelia contained in a piece of tissue could also be erroneously identified as follicles if histological analysis was not carried out.

Although the number of cultured follicles was small in the present study, it still implied that it was difficult to culture primordial and primary follicles individually. We have demonstrated in an earlier study that isolated primordial or primary follicles, and those which are surrounded only by a small amount of stromal tissue, do not survive and grow as well as follicles cultured within ovarian tissue slices (Hovatta et al., 1999).

Individual squamous cells were found in the aspirates from four patients, and this was consistent with the report by Artley et al. (Artley et al., 1993). They found squamous cells in cytological examination of fluid obtained from transvaginal aspiration of simple ovarian cysts, suggesting that squamous cells were carried from the vagina into the follicles by the aspiration needle as it passed through the vaginal wall. That is also the explanation for why a piece of tissue consisting of vaginal epithelium was found in follicular aspirate.

In conclusion, our data show that follicular aspirates obtained during oocyte retrieval in IVF units cannot be used as an efficient source of human follicles. Some structures found in the aspirate may be erroneously regarded as follicles.

Note added in proof

After the submission of our manuscript, Moskovtsev et al. reported similarly to us that only a few follicles could be found in follicular aspirates (Moskovtsev et al., 2002).

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References


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