Distinct microtubule and chromatin characteristics of human oocytes after failed in-vivo and in-vitro meiotic maturation

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BACKGROUND: While a complete failure of meiotic maturation following hCG administration is rare during IVF cycles, cases arise in which patients repeatedly display a high incidence of failure to complete maturation to metaphase II in vivo. For the immature oocytes of such patients, our objectives were (i) to ask whether progression to MII could be supported in vitro, and (ii) to define their microtubule/chromatin properties following in-vitro maturation (IVM). Together, these studies were aimed at augmenting our understanding of factors underlying meiotic arrest in the human. METHODS: Cases are presented here for two patients (A and B) producing oocytes that recurrently showed the inability to mature to metaphase II in vivo. Following IVM attempts, chromatin and microtubule characteristics were identified in those oocytes that remained arrested during meiosis I. RESULTS: In patient A, meiotically arrested oocytes exhibited clear defects in spindle and chromatin arrangements. In contrast, the majority of oocytes from patient B displayed normal MI and MII spindles with aligned chromosomes, although some oocytes exhibited indications for possible defects in cell cycle control. CONCLUSIONS: Together, these analyses illustrate two cases with oocytes exhibiting a common gross defect, that is meiotic maturation arrest, but revealing different aetiologies or manifestations as evidenced by the presence or absence of abnormal spindle/chromatin organization. This work reinforces the existence of intrinsic defects in oocytes of some patients, the molecular and cellular bases of which merit further investigation.

Key words: chromatin/human oocyte/in-vitro maturation/maturation arrest/spindle

Introduction

Oocytes acquire the ability to resume meiosis (meiotic competence) and to undergo normal embryonic development (developmental competence) in a sequential fashion during oogenesis. Upon receiving the normal stimulatory signal of pre-ovulatory LH in vivo, meiotically competent oocytes resume meiosis and proceed through meiosis I until arrest at metaphase of meiosis II. In human assisted reproductive technologies, ovarian stimulation protocols result in the development of multiple follicles and oocytes. However, a small proportion of oocytes fail to mature in vivo despite the use of exogenous hCG to induce the process of oocyte maturation. Typically during assisted conception cycles, and consistent with the experience in our programme, ~15% of oocytes fail to resume and/or to complete meiosis in response to hCG administration in vivo (Bar-Ami et al., 1994).

A complete or predominant failure to complete meiosis in vivo may also occur in rare instances. Several investigators have identified sporadic cases in which a whole cohort of oocytes failed to resume (persistent germinal vesicle) or complete meiosis (no polar body formation) despite hCG exposure (Neal et al., 2002); in some cases, such failure persisted even with attempts to stimulate maturation in vitro (Rudak et al., 1990; Harrison et al., 2000; Levran et al., 2002). Similarly, Hartshorne et al. (1999) described a case for which oocytes failed to mature in vivo or in vitro despite response to gonadotrophic stimulation as evidenced by normal follicular development and oocyte size. Harrison et al. (2000) and Levran et al. (2002) also reported cases with similar findings and observed arrest at metaphase I despite in-vitro maturation (IVM) attempts. Cases of maturation failure also appear to occur in a recurrent fashion (Hartshorne et al., 1999; Harrison et al., 2000; Bergere et al., 2001; Levran et al., 2002; Neal et al., 2002), the possibility being suggested that an inherited disorder exists in human oocytes (Schmiady and Neitzel, 2002).

While oocyte defects may help explain otherwise unexplained causes of infertility, the nature of oocyte maturation failures and their possible causes merit further investigation. It is conceivable that oocyte maturation anomalies possess different aetiologies. Indeed, an inability to respond to the
maturation signal(s) per se may lead to a failure to resume meiosis. Alternatively, intrinsic oocyte factors involving abnormal cell cycle control, spindle and/or cytoskeletal function may also contribute to failures in progression from metaphase I (MI) to metaphase II (MII). While previous reports have described failed maturation cases with, in certain cases, oocytes arresting at defined stages during oocyte maturation, a systematic analysis of the cytoskeleton has not been undertaken in these oocytes. Windt et al. (2001) were the first to document a case of meiotic arrest with abnormal spindles and dispersed chromosomes, and similar cytological aberrations were described in MI arrested oocytes from polycystic ovary patients (Jae et al., 2002).

In the present study, we describe cycles that were characterized by defects in the ability of oocytes to complete maturation in vivo and in vitro. Because of the known importance of the cytoskeleton and chromatin during the process of oocyte maturation, microtubule and chromatin markers were evaluated.

Materials and methods

Patients

Complete institutional review board approval and written consents were obtained prior to placing oocytes in culture. Findings from two patients are reported in the present analysis. Patient A was 32 years old with a primary diagnosis of endometriosis and day 3 FSH level of 11.1 mIU/ml; patient B was 30 years old with a primary diagnosis of uterine factor, and her day 3 FSH level was 8.0 mIU/ml.

Stimulation protocols

Controlled ovarian stimulation was performed using a variety of ovulation induction regimens. A low dose leuprolide lupron (LDLL), also known as a long lupon protocol, was employed in some cycles in which leuprolide acetate, a GnRH agonist (Lupron; TAP Pharmaceuticals, USA; 1.0 or 0.5 mg depending upon prior gonadotropin response) was begun either a week after documentation of urinary LH surge or the day after a mid-luteal progesterone determination, and was continued until at least day 2 of menses. Baseline ultrasonography and blood testing were then performed to document appropriate down-regulation. A variety of gonadotropin medications was used for ovulation induction, with Gonal F (Serono Laboratories, Inc., USA) or Folлизим (Organon, USA) as the FSH preparations, and Repronex (Ferring Pharmaceuticals, USA) as the hMG preparation. Standard stimulation was generally achieved using divided daily dosing of a total of 3–4 ampoules/day. Dosing was adjusted based upon patient age, FSH levels, and prior response to ovulation inductions. In a microflare lupron protocol (MicroFlr), 0.05 mg leuprolide acetate was begun on day 1 of the menstrual period following the oral contraceptives. If no cysts >3 cm were seen, gonadotrophin dosing of FSH and hMG was begun. Alternatively, the GnRH antagonist Antagon (Organon) was used to suppress gonadotrophin production; following gonadotrophin dosing of FSH and hMG, the antagonist was started on the morning of cycle day 7 to prevent the LH surge and was continued to the day of hCG administration. For all stimulation cycles, monitoring of follicle growth was achieved using ultrasound and measuring serum estradiol levels starting on stimulation day 6, and every 1–3 days thereafter, as indicated. A dose of 10 000 IU of hCG (Profasi; Serono) was administered i.m. when two follicles reached a maximum diameter of >20 mm (mean 16.5 mm) and with an estradiol concentration of >500 pg/ml. Transvaginal oocyte retrieval was performed.

Oocyte retrieval, evaluation and culture

Oocytes were retrieved by ultrasound-guided transvaginal aspiration 36 h after hCG administration except during the fourth cycle of patient B, in which oocyte retrieval was performed 39 h after hCG injection in an effort to increase the likelihood of obtaining mature oocytes. During their first two cycles, oocytes from both patients were inseminated with the partner’s sperm, and the meiotic stage of oocytes was evaluated at the fertilization check, 14–17 h post insemination. For their third attempts, fertilization was attempted using ICSI. About 3 h post-retrieval, oocytes were stripped of cumulus cells with hyaluronidase and mechanical pipetting. At this time, oocytes were initially assessed by gross morphological appearance as: GV (presence of an intact germinal vesicle), −PB (absence of a GV or a polar body), +PB (presence of a polar body) and Deg (degenerated oocyte). Within 2 h after removal of cumulus cells, +PB oocytes were injected with a single sperm, and immature oocytes (GV and −PB) were placed in culture for IVM within 6 h after oocyte retrieval.

Medium for IVM was prepared on the day prior to oocyte retrieval for overnight equilibration. In the first IVM attempt for both patients, Ham’s F10 medium (HF-10; Sigma Chemical Co., USA) supplemented with autologous, heat-inactivated follicular fluid (50:50, v/v) was used (Cha et al., 1991). On the fourth cycle of patient B, a different IVM culture medium was used: M199 medium supplemented with 10% synthetic serum substitute, 0.075 IU/ml rFSH, 0.075 IU/ml hCG, 1.0 μg/ml estradiol, 0.25 mmol/l sodium pyruvate, and 1 mmol/l glutamine was chosen (Chian et al., 1999; Cekleniak et al., 2001; Chian and Tan, 2002). Oocytes were assessed after 24–36 h in culture at which time IVM was terminated by fixing the oocytes.

Oocyte processing and analysis

Individual oocytes were fixed for 30 min at 37°C as previously described using a microtubule-stabilizing buffer containing 2% formaldehyde, 0.1% Triton X-100, 1 mmol/l taxol, 10 IU/ml aprotinin and 50% deuterium oxide (Combelles et al., 2002). Samples were then washed and stored in a blocking solution of phosphate-buffered saline containing 2% bovine serum albumin, 2% powdered milk, 2% normal goat serum, 0.1 mol/l glycine, and 0.01% Triton X-100 containing 0.2% sodium azide (PBS blocking solution) until processing. Oocytes were incubated for 3 h at 37°C with shaking with a mixture of monoclonal anti-α-tubulin and anti-β-tubulin (Sigma Biosciences, USA) at a 1:150 dilution, followed by three 15 min washes in PBS-blocking solution and a 90 min exposure to a 1:150 dilution of an affinity-purified fluorescein-labelled donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., USA). To visualize filamentous actin (f-actin), rhodamine-conjugated phallolidin (1 IU/ml, Molecular Probes Inc., USA) was included in the secondary antibody reagent. Following extensive washing, oocytes were incubated with 1 μg/ml Hoechst 33258 (Polysciences Inc., USA) in PBS blocking solution for 30 min at 37°C. Oocytes were mounted uncompressed in 50% glycerol/PBS containing sodium azide as an antifading reagent (25 mg/ml). Labelled oocytes were visualized using a Zeiss IM-35 inverted microscope, equipped with fluorescein and Hoechst selective filter sets and a 50 W mercury arc lamp using a ×63 Neofluor objective lens. Images were acquired for each oocyte using the ORCA II digital cooled CCD camera (Hamamatsu, USA) and the Metamorph image analysis software (Version 6.0; Universal Imaging Corporation, USA).
Results

Variables for all cycles for both patients undergoing IVF treatment at our centre are summarized in Table I, including type of stimulation, follicular response, estradiol (E2) levels, and oocyte parameters.

**Patient A**

During her three cycles at our centre, only immature oocytes were retrieved from this patient (Table I). Complete failure to achieve meiotic maturation occurred despite peak E2 levels in the 1100–4400 pg/ml range and the presence of numerous follicles >18 mm in diameter (Table I). During the third and last cycle, oocytes that were immature on the day of retrieval were placed in culture for IVM. Out of 17 oocytes retrieved, one oocyte was degenerate, and the remaining 16 oocytes that lacked a polar body were in-vitro matured. Oocyte diameters were within the normal range required to complete maturation in vitro (Durinzi et al., 1995; Combelles et al., 2002). After 29 h in culture, three out of 15 –PB oocytes matured to MII, and the single GV and one –PB oocyte became degenerate (Table II). ICSI was performed on the three MII oocytes from which complete fertilization failure resulted, with no pronuclear structures observed. The 11 viable –PB oocytes were fixed 29 h after the start of culture. Immunofluorescence analyses revealed that all 11 oocytes lacked a polar body and were meiotically arrested during the first division (Table II). Slightly more than half of these oocytes (6/11) displayed MI spindles approximating a bipolar arrangement, although the poles of the spindles were not tightly organized and ended abruptly, with some splaying of microtubule fibres (Figure 1A). In addition, chromosomes were not tightly organized at the spindle equator in any of these six oocytes; indeed, aberrations ranged from a couple of chromosomes displaced from the rest of them (two oocytes), to complete scattering of chromosomes within the spindle structure (four oocytes; Figure 1A). The remaining five –PB oocytes placed in culture failed to exhibit MI spindles with any evident sign of bipolarity (Figure 1B). Rather, multipolar masses of microtubules were observed, within which chromosomes were located. In none of these oocytes were microtubules observed, other than in association with chromosomes. It is also noted that in all oocytes, spindle structures had migrated to the cell periphery, with microfilaments found in the cortex but with a greater concentration in the area overlying the meiotic spindle.

**Patient B**

For all of treatment cycles at our centre, the oocytes of patient B displayed an elevated incidence of immature oocytes at the time of retrieval (81.8, 100, 72.7 and 40% of oocytes were immature at the time of retrieval (81.8, 100, 72.7 and 40% of oocytes were retrieved from this patient (Table I). Complete failure to achieve meiotic maturation occurred despite peak E2 levels in the 1100–4400 pg/ml range and the presence of numerous follicles >18 mm in diameter (Table I). During the third and last cycle, oocytes that were immature on the day of retrieval were placed in culture for IVM. Out of 17 oocytes retrieved, one oocyte was degenerate, and the remaining 16 oocytes that lacked a polar body were in-vitro matured. Oocyte diameters were within the normal range required to complete maturation in vitro (Durinzi et al., 1995; Combelles et al., 2002). After 29 h in culture, three out of 15 –PB oocytes matured to MII, and the single GV and one –PB oocyte became degenerate (Table II). ICSI was performed on the three MII oocytes from which complete fertilization failure resulted, with no pronuclear structures observed. The 11 viable –PB oocytes were fixed 29 h after the start of culture. Immunofluorescence analyses revealed that all 11 oocytes lacked a polar body and were meiotically arrested during the first division (Table II). Slightly more than half of these oocytes (6/11) displayed MI spindles approximating a bipolar arrangement, although the poles of the spindles were not tightly organized and ended abruptly, with some splaying of microtubule fibres (Figure 1A). In addition, chromosomes were not tightly organized at the spindle equator in any of these six oocytes; indeed, aberrations ranged from a couple of chromosomes displaced from the rest of them (two oocytes), to complete scattering of chromosomes within the spindle structure (four oocytes; Figure 1A). The remaining five –PB oocytes placed in culture failed to exhibit MI spindles with any evident sign of bipolarity (Figure 1B). Rather, multipolar masses of microtubules were observed, within which chromosomes were located. In none of these oocytes were microtubules observed, other than in association with chromosomes. It is also noted that in all oocytes, spindle structures had migrated to the cell periphery, with microfilaments found in the cortex but with a greater concentration in the area overlying the meiotic spindle.

**Table I.** Cycle characteristics for patients A and B

<table>
<thead>
<tr>
<th>Patient</th>
<th>Cycle</th>
<th>Stimulation*</th>
<th>Estradiol (pg/ml)</th>
<th>Follicles</th>
<th>Oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1</td>
<td>LDL/FSH</td>
<td>1148</td>
<td>11</td>
<td>11F</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(day 12)</td>
<td>6 follicles &gt;18 mm</td>
<td>11 –PB</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5 follicles &lt;18 mm</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>2</td>
<td>Antagonist/</td>
<td>2149</td>
<td>19</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HMG/FSH</td>
<td>(day 12)</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10 follicles &gt;18 mm</td>
<td>1 GV, 9 –PB</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9 follicles &lt;18 mm</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>3b</td>
<td>MicroFlrL/</td>
<td>4390</td>
<td>21</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HMG/FSH</td>
<td>(day 12)</td>
<td></td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>16 follicles &gt;18 mm</td>
<td>1 GV, 15 –PB, 1 deg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5 follicles &lt;18 mm</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>LDL/FSH</td>
<td>1821</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(day 11)</td>
<td>7 follicles &gt;18 mm</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5 follicles &lt;18 mm</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>LDL/FSH</td>
<td>2128</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(day 11)</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5 follicles &gt;18 mm</td>
<td>1 GV, 7 –PB, 1 deg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5 follicles &lt;18 mm</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>3b</td>
<td>Antagonist/</td>
<td>1763</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HMG/FSH</td>
<td>(day 12)</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7 follicles &gt;18 mm</td>
<td>1 GV, 7 –PB, 3 +PB, 1 deg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td>5 follicles &lt;18 mm</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>4b</td>
<td>Antagonist/</td>
<td>1106</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HMG/FSH</td>
<td>(day 14)</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3 follicles &gt;18 mm</td>
<td>1 GV, 1 –PB, 3 +PB</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5 follicles &lt;18 mm</td>
<td></td>
</tr>
</tbody>
</table>

Estradiol levels on the day of hCG administration are shown with the day in the stimulation cycle in parentheses.

*Stimulation protocols: see Materials and methods for detailed description.

*Cycles for which in-vitro maturation was attempted and oocytes were fixed for immunocytochemistry. The total number of follicles with a diameter of >12 mm is given on the day of hCG administration. The total number of oocytes retrieved post-hCG are indicated for each cycle with meiotic stages assessed by morphological criteria.

LDLL = low dose leuprolide lupron; FlrL = microflare lupron protocol; GV = presence of a germinal vesicle; –PB = absence of a polar body; +PB = presence of a polar body; deg = degenerate.

**Table II.** Meiotic stages of oocytes following in-vitro maturation (IVM) using immunofluorescence analysis for assessment

<table>
<thead>
<tr>
<th>Patient</th>
<th>Cycle</th>
<th>Hours</th>
<th>At the start of IVM</th>
<th>At the end of IVM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>GV –PB</td>
<td>GV –PB</td>
</tr>
<tr>
<td>A</td>
<td>3</td>
<td>29</td>
<td>1 15</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>3</td>
<td>24</td>
<td>1 7</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>36</td>
<td>0 2</td>
<td>0</td>
</tr>
</tbody>
</table>

GV = germinal vesicle; PB = polar body; Deg. = degenerate.
Meiotic block in human oocytes

variety of patterns observed for patients A (A, B, a, b) and B (C, D, c, d). (A, a) Spindle structure with associated chromosomes in a meiosis I arrested oocyte from patient A; note that although the spindle displayed two polar regions, these did not appear as focused ends, and chromosomes were dispersed within the spindle. In (B, b), the other category of spindle/chromatin organization which was observed in meiosis I arrested oocytes from patient A is displayed. At the time of observation, the spindle structure completely lacked any signs of bipolarity, being merely composed of a tightly organized microtubule mass within which chromosomes were located. (C, c) Metaphase I spindle characteristic of meiotically arrested oocytes from patient B; spindles were bipolar with chromosomes aligned at the equatorial region. As shown in (D, d) for patient B, normal bipolar spindles with aligned chromosomes were also observed in metaphase II oocytes after in-vitro maturation. pb = polar body. Scale bar = 10 μm.

Figure 1. Organization of microtubules and chromosomes in oocytes following in-vitro maturation in two patients with meiotic maturation failure. Correlative total α/β-tubulin (A, B, C, D) and chromatin (a, b, c, d) staining in in-vitro matured oocytes demonstrate the range of patterns observed for patients A (A, B; a, b) and B (C, D; c, d). (A, a) Spindle structure with associated chromosomes in a meiosis I arrested oocyte from patient A; note that although the spindle displayed two polar regions, these did not appear as focused ends, and chromosomes were dispersed within the spindle. In (B, b), the other category of spindle/chromatin organization which was observed in meiosis I arrested oocytes from patient A is displayed. At the time of observation, the spindle structure completely lacked any signs of bipolarity, being merely composed of a tightly organized microtubule mass within which chromosomes were located. (C, c) Metaphase I spindle characteristic of meiotically arrested oocytes from patient B; spindles were bipolar with chromosomes aligned at the equatorial region. As shown in (D, d) for patient B, normal bipolar spindles with aligned chromosomes were also observed in metaphase II oocytes after in-vitro maturation. pb = polar body. Scale bar = 10 μm.

Immature for cycles 1–4; Table II). Slightly modified stimulation protocols were used for each cycle, with the duration varying from 11 to 14 days and peak E2 levels ranging 1100 to 2100 pg/ml (Table I). On the day of the hCG trigger, three to seven follicles >18 mm in diameter were measured for each cycle (Table I). Given the high proportion of immature oocytes during her first and second cycles, the immature oocytes retrieved in her third cycle were placed in IVM culture in an attempt to increase the number of mature MII oocytes available for ICSI. In this third cycle, patient B possessed eight immature oocytes at the time of retrieval, with one GV stage oocyte and seven oocytes with no visible polar body (Table II). After IVM for 24 h, the oocyte that was a GV at the start of culture remained in the GV stage, and the other seven oocytes still lacked a polar body by gross morphological examination (Table II). Immunofluorescence analysis revealed that, in addition to a rimmed nucleolus, the chromatin of the GV stage oocyte was disposed as threads throughout the nucleus; this was characteristic of a chromatin pattern defined previously by our group to represent oocytes that are incapable of resuming meiosis in vitro (Combelles et al., 2002). Analysis of the seven −PB oocytes allowed us to define the precise stages at which meiotic arrest occurred; three oocytes lacked a polar body while four oocytes possessed a polar body (Table II). However, the polar bodies were unusually small and formed a deep invagination into the oolemma, making them difficult to discern by gross morphological examination. Phalloidin staining revealed a cortex rich in f-actin, in particular in the region overlying the meiotic spindle near the cell surface. Nevertheless, immunofluorescence analysis revealed that the spindles were organized normally, with bipolar spindle structures and aligned chromosomes documented in 100% of −PB (4/4; Figure 1C, c) and −PB (3/3; Figure 1D, d) oocytes. In addition to the above eight immature oocytes placed in culture for IVM, three mature MII oocytes were obtained following oocyte retrieval. After ICSI, two out of the three injected oocytes exhibited two pronuclei, subsequently giving rise to one embryo arrested at the 1-cell stage and one poor quality 6-cell embryo. Both of these embryos were transferred per request of the patient, and a chemical pregnancy resulted.

Because four out of seven −PB oocytes progressed to MII with apparently normal cytological features after IVM, and a chemical pregnancy had occurred in cycle 3, patient B chose the option to undergo a fourth cycle in combination with IVM. In addition, in an effort to increase the proportion of mature oocytes, oocyte retrieval was delayed by 3 h, being performed at 39 h post-hCG. Following retrieval, six cumulus–oocyte complexes (COC) were retrieved, with five COC destined for ICSI and one COC set aside for IVF. In addition, a late insemination paradigm was included in this cycle to allow any oocytes that had failed to complete maturation in vitro to do so in vitro in the presence of surrounding cumulus cells. Due to the low number of COC retrieved, only a single oocyte was inseminated with the partner’s sperm 10 h post-retrieval (as opposed to our normal insemination time of 4–6 h post-retrieval). At 19 h post-insemination, this oocyte was confirmed to possess a single polar body with no pronuclei. Fixation and immunofluorescence analysis documented that the unfertilized oocyte was arrested in MII with a normal bipolar spindle and aligned chromosomes, with a lack of sperm penetration into the

Because four out of seven −PB oocytes progressed to MII with apparently normal cytological features after IVM, and a chemical pregnancy had occurred in cycle 3, patient B chose the option to undergo a fourth cycle in combination with IVM. In addition, in an effort to increase the proportion of mature oocytes, oocyte retrieval was delayed by 3 h, being performed at 39 h post-hCG. Following retrieval, six cumulus–oocyte complexes (COC) were retrieved, with five COC destined for ICSI and one COC set aside for IVF. In addition, a late insemination paradigm was included in this cycle to allow any oocytes that had failed to complete maturation in vitro to do so in vitro in the presence of surrounding cumulus cells. Due to the low number of COC retrieved, only a single oocyte was inseminated with the partner’s sperm 10 h post-retrieval (as opposed to our normal insemination time of 4–6 h post-retrieval). At 19 h post-insemination, this oocyte was confirmed to possess a single polar body with no pronuclei. Fixation and immunofluorescence analysis documented that the unfertilized oocyte was arrested in MII with a normal bipolar spindle and aligned chromosomes, with a lack of sperm penetration into the
patients exclusively with unexplained infertility (Eichenlaub-Ritter et al., 1995), these studies strictly pertained to the post-ovulatory ageing of MII oocytes (Eichenlaub-Ritter et al., 1988; Pickering et al., 1988). Characteristics of ageing in human oocytes include microtubules emanating from the spindle surface, spindle collapse, stray and dispersed chromosomes, and cytoplasmic microtubules (Eichenlaub-Ritter et al., 1988; Pickering et al., 1988; Eichenlaub-Ritter et al., 1995). Importantly, in none of the oocytes from patient A were any of these characteristics identified. The known association between an elevated incidence of spindle abnormalities and advanced maternal ageing (Battaglia et al., 1996; Volarcik et al., 1998) is also difficult to relate here, given that the age of patient A was 32 years. In contrast to our findings, Windt et al. (2001) reported spindle aberrations in cases of oocyte maturation disorders, with a complete absence of microtubules from the spindle region and dispersion of the chromosomes. Conceivably, intrinsic differences may exist between patients in their ability to form and/or maintain stable spindle structures. Mechanisms underlying meiotic spindle formation and function are poorly understood with the exact roles of chromosomes, centrosomes and motor proteins remaining ill-defined, in particular in human oocytes (Carabatosos et al., 2000; Combelles et al., 2002). While yet unknown structural defects may explain an absence of spindle bipolarity in patient A, our limited knowledge of spindle assembly determinants in human oocytes precludes one from pinpointing the deficiency in patient A.

Clearly, the meiotic arrest and spindle modifications described in this study may be caused by a multitude of defects. Oocytes that have not completed the growth phase of oogenesis have not yet acquired full meiotic competence; indeed, these oocytes typically resume meiosis and arrest at metaphase of meiosis I (Sorensen and Wassarman, 1976). It would be difficult to conclude that the immature oocytes analysed in the present study were still in the growing phase of oogenesis given that follicular sizes were within normal ranges and were accompanied by typical peak estradiol levels across multiple cycles for both patients (Table 1). Furthermore, normal oocyte diameters and apparent maturity of the corona were observed (data not shown). However, one cannot exclude the possibility that the function of the follicular cells was compromised, thereby resulting in aberrant communication and signalling pathways between the somatic and germ cell compartments. Notably, altered control mechanisms, even in the final stages of oocyte maturation, have been associated with an increase in congression failure with its associated risk in non-disjunction error (Hodges et al., 2002). Whether similar intraovarian deficiencies explain the defects presented here remains to be determined. In an attempt to rescue MI arrest, IVM resulted in a limited number of MII oocytes, albeit of suboptimal cytoplasmic quality and developmental potential. While the use of IVM in clinical assisted reproduction treatment remains experimental and awaits significant optimization, clear deficits that persisted over time in vivo were observed here. Thus, our report, together with the work of others, suggests the existence of a genetic disorder in which
human oocytes are incapable of completing meiosis (Eichenlaub-Ritter et al., 1995; Schmiady and Neitzel, 2002). Lastly, it is noted that environmental exposures may also affect the oocyte and, more precisely, the meiotic process and spindle arrangements (Yin et al., 1998; Hunt et al., 2003).

Oocytes appear to possess unique controls for key cell cycle transitions, which differ from checkpoints known to exist in somatic cells (LeMaire-Adkins et al., 1997; Fulka et al., 1998). While a chromosome alignment checkpoint may not be in place in oocytes, the present report lends credence to the possible existence of a spindle assembly checkpoint. In addition, the finding that progression into anaphase of meiosis I is dependent upon post-translational modifications of kinetochore elements (Brunet et al., 1999) may represent a permissive chromosome alignment checkpoint, possibly reflected in the meiotic failure case(s) presented here. Given a known requirement for microtubules and intact spindle structures in meiotic transitions (Kubiak et al., 1993), spindle defects may preclude normal meiotic progression in patient A. Lastly, it is noted that the presence of actin filaments in the oocyte cortex and the formation of an actin-rich domain, together with normal spindle migration, preclude the possibility that disturbances in actin polymerization/distribution could explain meiotic arrest in patients A and B (Maro et al., 1986; Kim et al., 1998).

A previously described genetic defect in LT and LTXBO strains of mice includes MI arrest and an elevated incidence of spontaneous parthenogenetic activation, even when oocytes are fully grown (Hampl and Eppig, 1995; Hiroa and Eppig, 1997; 1999; Ciemerych and Kubiak, 1998). In this mouse model, deficiencies were identified at the level of cell cycle control mechanisms such that activity of the maturation-promoting factor (MPF) was sustained with a failure to degrade cyclin B despite the existence of normal spindle structures (Hampl and Eppig, 1995). More than one defect is thought to exist in LT oocytes, with intrinsic deficits in the ability to trigger anaphase (Hiroa and Eppig, 1999; Eppig et al., 2000). With multiple types of cell cycle kinases implicated in cell cycle control during oocyte maturation, it remains to be determined whether similar or related biochemical defects exist in human oocytes.

In conclusion, the present immunofluorescence analyses of immature oocytes from two patients have shown that while both patients exhibited a gross failure to complete meiosis in vivo, different aetiologies may underlie comparable manifestations. However, a better understanding of the various possible underlying defects ought to be attained so that efforts can be directed towards the development of a precise diagnosis along with a treatment option for such deficiencies. In the meantime, while awaiting such advances, the present work supports the use of microtubule and chromatin examination to identify intrinsic oocyte defects. As a result, patients can be better counselled and guided towards an oocyte donation cycle, most importantly, at an earlier time during their infertility treatment.

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