Developmental ability of human oocytes with or without birefringent spindles imaged by Polscope before insemination

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BACKGROUND: Birefringent spindles imaged with the Polscope can predict fertilization rates after intracytoplasmic sperm injection (ICSI). The present study examined the development of human oocytes with or without birefringent spindles, imaged with the Polscope before ICSI. METHODS: Oocytes were obtained from stimulated ovaries of consenting patients undergoing oocyte retrieval for ICSI. Spindles were imaged with the Polscope combined with a computerized image analysis system. After imaging and ICSI, oocytes with or without spindles were cultured separately for examination of fertilization and embryo development. A total of 1544 oocytes from 136 cycles were examined with the Polscope and inseminated by ICSI. RESULTS: Spindles were imaged in 82% of oocytes. After ICSI, more oocytes ($P < 0.05$) with spindles (69.4%) fertilized normally, forming 2 pronuclei, than oocytes without spindles (62.9%). At day 3, more oocytes ($P < 0.01$) with spindles (66.3%) developed to 4–11 cell stages than oocytes without spindles (55.4%). Significantly more ($P < 0.001$) oocytes with spindles developed to morula and blastocyst by day 5 (51.1 versus 30.5%) and day 6 (53.2 versus 29.3%) compared with oocytes without spindles. CONCLUSIONS: The results indicate that the presence of a birefringent spindle in human oocytes can predict not only higher fertilization rate, but also higher embryo developmental competence.

Key words: embryo development/human oocytes/Polscope/spindles

Introduction

Meiotic spindles are composed of microtubules and are crucial for normal chromosome alignment and separation of maternal chromosomes during meiosis I and meiosis II. Disruption of the meiotic spindle results in abnormal chromosome alignment. Fertilization of such oocytes usually results in aneuploid embryos. A recent study by Battaglia et al. indicated that the proportion of oocytes with abnormal meiotic spindles was significantly higher in older than younger women, and that abnormal spindles may contribute to the high prevalence of aneuploidy in embryos from this population (Battaglia et al., 1996). Moreover, suboptimal in-vitro manipulation, such as fluctuation of temperature (Pickering et al., 1990; Almeida and Bolton, 1995), has also been shown to disrupt spindle structure and therefore contribute to aneuploidy. Aneuploid embryos may develop to early, or even blastocyst stage (Munné et al., 1995), thus presumably some transferred embryos are aneuploid. This may be one of the reasons that the pregnancy rate is lower in older women.

Since most aneuploidies arise in the oocytes, studies of oocyte and particularly spindle structure, might help identify normal from abnormal embryos. Until now, no satisfactory method existed for clinical IVF to distinguish morphologically normal spindles. Conventional methods to image the spindle rely on fixation (Pickering et al., 1988; Pickering et al., 1990; Almeida and Bolton, 1995; Battaglia et al., 1996), so they provide limited value to clinical IVF. Recently, we found that spindles in living human oocytes could be imaged with a new orientation-independent polarized microscope (Polscope) (Wang et al., 2001). The Polscope employs novel electro-optical hardware and digital processing to enhance the sensitivity of polarized optics to image macromolecular structures within cells based on their birefringence (Oldenburge, 1999). Since it illuminates specimens with the same intensity of light as differential interference contrast (DIC), and since DIC, which also employs a form of polarized light, has been used safely during IVF for over 20 years, the Polscope should not be detrimental to human oocytes. We have demonstrated no detrimental effects on mouse (Liu et al., 2000) and human (W.H.Wang et al., unpublished data) oocytes and embryos when they were exposed to the Polscope, so the Polscope has potential for application in human IVF. Our previous studies
Figure 1. Human oocytes imaged with DIC (A and B) and with the Polscope (A’ and B’). (A) and (A’) show an oocyte with birefringent spindle which was not visible with DIC optics (A) but was imaged with the Polscope (A’). The spindle is located just under the plasma membrane where the polar body (Pb) was released. (B) and (B’) show an oocyte without spindle birefringence imaged with both DIC optics (B) and the Polscope (B’). Original magnification, ×350.

Table I. Fertilization and embryo development of human oocytes with or without birefringent spindle. Values in parentheses are percentages

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Spindle</th>
<th>No. of oocytes</th>
<th>No. of oocytes with 2PN</th>
<th>No. and stages of viable embryos on day 3</th>
<th>Day 5 embryos&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Day 6 embryos&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total&lt;sup&gt;f&lt;/sup&gt;</td>
<td>4–7cell&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8–11cell&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>136</td>
<td>Yes</td>
<td>1266 (82.0)</td>
<td>879 (69.4)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>583 (66.3)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>433 (74.3)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>150 (25.7)&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>278 (18.0)</td>
<td>175 (62.9)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>97 (55.4)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>81 (83.5)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>16 (16.5)&lt;sup&gt;f&lt;/sup&gt;</td>
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</table>

<sup>a</sup>Data from 63 cycles.
<sup>b</sup>Data from 41 cycles.
<sup>c</sup>Percentage of number of oocytes with 2PN.
<sup>d</sup>Percentage of total number of viable embryos on day 3.
<sup>e</sup>No. of blastocysts and morulae/no. of 2 PN oocytes (from 63 cycles on day 5 and 41 cycles on day 6. For more details, see experimental designs).
<sup>f</sup>Values with different superscripts in the same column are different significantly (f<sub>g</sub>P < 0.05; h<i>P < 0.01; j<k>P < 0.001).

PN = pronuclei.

also indicated that birefringent spindles in living human oocytes predicted higher fertilization rates after ICSI (Wang et al., 2001) and that human spindles were easily destroyed during in-vitro manipulation (Wang et al., 2000a) or during oocyte meiotic maturation (Wang et al., 2000b). Therefore, changes in spindle structure may reflect cytoplasmic dysfunction or other damage to the oocytes. In the present study, we examined our hypothesis that spindle structure may predict the rate of embryo development in vitro.

Materials and methods

Sources of oocytes

Approval was obtained from the Women and Infants Hospital Institutional Review Committee to study unfertilized human oocytes and to study images of oocytes obtained during human IVF. Oocytes were from stimulated ovaries of consenting patients undergoing oocyte retrieval for ICSI. After retrieval, oocytes were cultured in P1 medium (Irvine Scientific, Santa Ana, CA, USA) containing 6% synthesized serum substitute (SSS; Irvine Scientific) for 5–6 h. Before examination
with the Polscope, cumulus cells were removed by pipetting in modified human tubal fluid (HTF) (Irvine Scientific) containing 80 IU/ml hyaluronidase (Sigma Chemical Co., St Louis, MO, USA).

**Spindle examination in oocytes with the Polscope and ICSI**
For imaging spindles and performing ICSI, each oocyte was placed in a 5 µl drop of modified HTF covered with warm paraffin oil (Gallard-Schleserger, Coral Place, NY, USA) in a Biotechs Delta T.C.O. Culture Dish System (Biotechs Inc., Butler, PA, USA). The system comprises a temperature controller, a stage adapter and the Delta T culture dish that has a specially coated clear glass (0.15 mm thick) bottom. The dishes were maintained at 37 ± 0.5°C during examination and ICSI. Oocytes were examined under a Zeiss Axiovert 100 with a Neofluar ×40 strain-free objective with a Biotechs Objective Heating System (Biotechs Inc.) and LC Polscope optics and controller (CRI, Cambridge, MA, USA), combined with a computerized image analysis system (MetaMorph Universal Imaging System, West Chester, PA, USA). After imaging, ICSI was performed. Since the first polar body position in many oocytes does not predict accurately the spindle position (Wang et al., 2001), in oocytes with spindles, ICSI was performed after oocytes were rotated to place the spindle at the 6 or 12 o’clock position relative to the injection needle. In oocytes without birefringent spindles, ICSI was performed after the first polar body was placed at 6 or 12 o’clock relative to the injection needle. After ICSI, oocytes with or without spindles were washed and cultured separately in P1 medium (Irvine Scientific) supplemented with 6% SSS for examination of fertilization.

**Embryo culture and examination**
Sixteen to 18 h after ICSI, fertilization was evaluated and oocytes with 2 pronuclei and a second polar body were considered as normally fertilized. Fertilized oocytes were washed 3–4 times and cultured in freshly prepared growth medium (P1 + 10% SSS), which had been equilibrated in a CO₂ incubator (3% CO₂ in air, 37°C) overnight. On day 3 (the day of examination of fertilization was considered to be day 1), embryo cleavage and viability were evaluated according to laboratory protocols. If patients had fewer than seven fertilized oocytes, embryo transfer was conducted on day 3 and the remaining embryos cultured until day 5 or day 6, with excess viable embryos being frozen. If patients had seven or more normal fertilized oocytes, embryos were transferred on day 3 to blastocyst medium (Irvine Scientific) containing 10% SSS until embryo transfer on day 5 or day 6.

**Experimental design**
Imaging of birefringent spindles with the Polscope, ICSI on day 0 (oocyte retrieval day), fertilization examination on day 1 (16–18 h after ICSI) and cleavage evaluation on day 3 were conducted on all oocytes from 136 cycles. As 73 patients received embryo transfer on day 3, no embryo evaluation was available on days 5 and 6 for these cycles. The 63 patients who received embryo transfer on day 5 (22 cycles) and day 6 (41 cycles) were evaluated on days 5 and 6 respectively.

**Statistical analysis**
Comparisons of data between oocytes with or without spindles were conducted by χ² test. Probability of P < 0.05 was considered to be statistically significant.

**Results**

**Spindles in living human oocytes imaged with the Polscope**
As shown in Figure 1, when oocytes were examined under DIC optics, no differences in the oocyte morphology were discernable (Figure 1A and B), except in rare cases when oocytes were grossly degenerated. However, when the same oocytes were examined with the Polscope, birefringent spindles were imaged and their location relative to the first polar body were identified (Figure 1A’). For this study, oocytes were divided into two groups based on the presence or absence of birefringent spindles. A total of 1544 oocytes from 136 cycles were examined with the Polscope, and spindles were imaged in 82% of oocytes (Figure 1A’). Birefringent spindles were not imaged in the remaining 18% of oocytes (Figure 1B’).

**Relationship between birefringent spindles and fertilization and development rates after ICSI**
As shown in Table I, after ICSI, more (P < 0.05) oocytes with spindles (69.4%) were fertilized normally, forming 2 pronuclei and releasing the second polar body, than oocytes without spindles (62.9%). On day 3, more (P < 0.01) oocytes with spindles developed normally (66.3%) than oocytes without spindles (55.4%). More (P < 0.05) oocytes with spindles developed to advanced cell stages (8- to 11-cell) than oocytes without spindles. More (P < 0.001) morulae and blastocysts formed from oocytes which had exhibited spindles than oocytes without spindles on day 5 (51.1 versus 30.3%) and on day 6 (53.2 versus 29.3%). As shown in Figure 2, the proportions of oocytes with birefringent spindles were 84.3, 75.1, 82.3 and 75% for patients aged <35 (74 cycles), 35–37 (26 cycles), 38–40 (32 cycles) and >40 years (4 cycles) respectively, with no differences observed between different age groups. However, the average number of oocytes imaged was significantly fewer in the patients at the age of >40 (4.0 ± 1.22) than those at the ages of <35 (11.9 ± 6.98), 35–37 (11.3 ± 6.37) and 38–40 years (11.0 ± 6.4).

**Discussion**
Results obtained in the present study indicate that spindle birefringence in living human oocytes can be imaged with the
Polscope, and that the presence of a meiotic spindle predicts not only a higher fertilization rate, but also a higher embryo development rate, thus suggesting that the presence of birefringent spindles in human oocytes predicts oocyte quality.

Spindle birefringence is an inherent physical property of the microtubule based structure. In contrast to fluorescence microscope imaging of spindles, polarized light imaging does not require invasive preparative techniques, such as fixation or staining. In addition to providing information about the presence or absence of the spindles, the Polscope also gives quantitative information about the morphological structure of the spindles, which was not analysed in this study. Further studies will need to evaluate the value of this quantitative, morphological information on spindle birefringence. Spindle structure may reflect oocyte quality by serving as a marker for reproductive senescence, cytoplasmic maturation and/or of pH and/or temperature stress during handling.

What induces embryo developmental blocks in oocytes lacking a spindle is unknown. Some explanations for the low developmental ability of oocytes without birefringent spindles can be suggested. First, it has been estimated that around a quarter of human oocytes from stimulated cycles are aneuploid and the frequency of this abnormality increases with age (Janny and Ménezo, 1996). Early studies indicated that genomic activation was necessary for blastocyst formation in the human. Failure of genetic activation may result in arrest of human embryos at the 8-cell to morula stages (Tesarik, 1989). In this study, we found that oocytes without spindles during imaging released the second polar body if they were fertilized. These results may suggest that oocytes did not show a spindle due to temporary depolymerization of microtubules by some environmental changes, such as temperature (Wang et al., 2000a). However, these oocytes recovered the spindles after being returned to the incubator. We do not know whether these oocytes will be aneuploid after fertilization. It is possible that oocytes without birefringent spindles may have abundant chromosomal abnormalities, which in turn might induce cell cycle arrest although direct confirmation of the association between aneuploidy and spindle structure is needed.

Secondly, because the first polar body position does not always accurately predict the spindle position (Wang et al., 2001), the damage to the spindles caused by ICSI is more likely to occur in oocytes without visible spindles.

Finally, oocytes without a birefringent spindle may be of poorer quality, thus developing also poorly as compared with those with birefringent spindles.

Battaglia et al. reported that the proportion of oocytes with abnormal spindles was significantly higher in older than in younger women, and abnormal spindles were associated with abnormal chromosome distribution (Battaglia et al., 1996). In the present study, although we found patient-dependent effects on the proportion of oocytes with or without birefringent spindles, we did not find any relationship between birefringent spindles and patient age. The difference between our study and that of Battaglia et al. 1996, in addition to the methods employed to image spindles, is that they obtained oocytes from ‘normal volunteers’ while all our oocytes were retrieved from infertile patients. This is presumably why many young infertile patients may suffer spindle abnormalities. Other factors may also have influenced the presence of spindle birefringence in our study. For example, as the cytoskeleton is quite sensitive to environmental changes (Pickering et al., 1988; Pickering et al., 1990; Almeida and Bolton, 1995; Wang et al., 2000a), disruption of spindle architecture may have arisen during in-vitro manipulation. Also, the small number of cycles from older patient limits the ability to make conclusions about the effects of maternal age on spindle birefringence.

In the present study, we used objective heater in the X40 objective lens during oocyte examination and ICSI to maintain the medium temperature at 37°C, which we found to improve spindle stability and embryo quality. Despite this, a proportion of oocytes (<20%) still did not show birefringent spindles. As mentioned above, imaging of spindles in living oocytes under the Polscope is based on birefringence, an inherent physical property of the microtubules. Thus, oocytes not exhibiting spindle birefringence probably sustained insult during oocyte development, maturation and/or other in-vitro conditions. Furthermore, oocyte age, maternal age and other patient-dependent factors may disrupt spindles.

In this study, we found that the outcome of oocytes with spindles (from fertilization to blastocyst formation) were superior to those of oocytes without spindles. It is possible that such a tendency continues during subsequent development during implantation and pregnancy, although further studies are needed. It also is not clear at present whether decreased development in oocytes without spindles is related specifically to aneuploidies, so future studies should examine this possibility, since the spindle plays such a crucial role during meiotic maturation and fertilization.

In conclusion, our results indicate that spindle birefringence can predict fertilization and embryo development. Further studies are needed to examine the relationship between spindle birefringence and the formation of aneuploidies and also to examine whether quantitative assessment of spindle structure and/or birefringence can further predict embryo development as well as pregnancies.

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References
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