

# Rigorous thermal control during intracytoplasmic sperm injection stabilizes the meiotic spindle and improves fertilization and pregnancy rates

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**Objective:** To examine the effects of different thermodynamic control systems on the temperature stability of human eggs during in vitro manipulation, with the integrity of meiotic spindles imaged using the LC-PolScope (Cambridge Research & Instrumentation, Inc., Woburn, MA).

**Design:** We performed intracytoplasmic sperm injection (ICSI) and/or imaging of eggs with the temperature regulated by three different systems: thermostated coverslip (system 1), thermostated coverslip combined with objective heater (system 2), and conventional stage warmer (system 3).

**Setting:** Academic in vitro fertilization clinic.

**Patient(s):** Oocytes were aspirated from stimulated ovaries of patients undergoing oocyte retrieval for ICSI.

**Intervention(s):** Measurement of temperature regulation in media surrounding eggs during in vitro manipulation and imaging.

**Main Outcome Measure(s):** Rate of oocytes with spindles, fertilization rates, and clinical pregnancy rates after ICSI.

**Result(s):** We imaged spindles in more oocytes with system 2 (81.2%) than with system 1 (61.4%). Spindles could not be imaged for system 3 because of technical limitations. Fertilization rates were significantly higher when oocytes were imaged and used for ICSI with system 2 (78.8%) than with system 1 (56.7%) or system 3 (64.0%). Most importantly, a significantly higher clinical pregnancy rate was observed when oocytes were manipulated with system 2 (51.7%) than with system 1 (25.0%) or system 3 (23.1%). No differences were found in average ages, number of previous cycles, number of eggs, or day 3 FSH or E<sub>2</sub> levels among groups.

**Conclusion(s):** Imaging meiotic spindles with the PolScope provides an intracellular thermostat during ICSI. Rigorous thermal control during ICSI stabilized spindles and increased the fertilization and clinical pregnancy rates achieved after ICSI. The presence of birefringent spindles in living human eggs served as a monitor for in vitro conditions. (Fertil Steril® 2002;77:1274–7. ©2002 by American Society for Reproductive Medicine.)

**Key Words:** Human oocytes, ICSI, spindle, PolScope, temperature stabilization

Meiotic spindles are formed from microtubules composed of polymerized tubulin. They are exquisitely sensitive to fluctuations in temperature (1–4). In vitro fertilization (IVF) offers the opportunity to study the role of the meiotic spindle in human oocyte developmental potential, because oocytes are ovulated at the metaphase II stage when the chromosomes are poised on the metaphase plate, tethered by microtubules that are inherently unstable relative to other structures in the oocyte. Unfortunately, imaging methods currently used in the IVF laboratory (i.e., Hoffman, Nomarski, or

bright field microscopy) cannot image clearly the meiotic spindle from human oocytes. Traditional methods to image spindles depend on fixation and immunostaining (1, 2, 5), so they provide limited value to clinical IVF.

Recently, we found that when an orientation-independent polarized light microscope (LC-PolScope, Cambridge Research & Instrumentation, Inc., Woburn, MA) was used to examine eggs, spindles could be imaged in the oocytes of the hamster (6), mouse (7), and human (8, 9). The PolScope employs novel

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## MATERIALS AND METHODS

### Sources of Oocytes

electrooptical hardware and digital processing to image macromolecular structures within cells based on their birefringence (10). The birefringence of the spindle occurs naturally. In contrast to fluorescence microscopy, polarized light imaging does not require invasive preparative techniques such as fixation and staining. The PolScope also provides the advantage of measuring both architecture and morphologic arrangements of spindles (10).

Approval was obtained from the Women and Infants Hospital Institutional Review Committee to study images of oocytes obtained during human IVF. Oocytes were aspirated from stimulated ovaries of consenting patients who were undergoing oocyte retrieval for ICSI. After retrieval, oocytes were cultured in P1 medium (Irvine Scientific, Santa Ana, CA) containing 6% synthesized serum substitute (SSS; Irvine Scientific) for 5 to 6 hours. Before the examination with the PolScope and the process of ICSI, cumulus cells were removed by pipetting in modified human tubal fluid (HTF) (Irvine Scientific) containing 80 IU/mL of hyaluronidase (Sigma Chemical Co., St. Louis, MO). Oocytes obtained from patients between December 1998 and December 1999 were randomly chosen for imaging and/or ICSI with three different warming systems.

In a previous study, we found that the PolScope could be used to monitor spindle position during intracytoplasmic sperm injection (ICSI). As the first polar body position did not always accurately predict spindle position (8), the PolScope could be used to avoid disruption of spindles during ICSI. We also found that the presence of a birefringent spindle could predict higher fertilization (8) and early developmental rates (9). However, when we examined human eggs during conventional ICSI in women, a high proportion of oocytes exhibited abnormal or missing spindles, and the oocytes with abnormal spindles had decreased fertilization rates (8). It is well known that spindles in human oocytes are exquisitely sensitive to environmental changes, such as fluctuation of temperature (1–4), so disassembled spindles in some oocytes may arise from changes in temperature. It also is possible that abnormal spindles are formed during oocyte meiotic maturation, as reported by Battaglia et al. (5). It seems that spindles in human oocytes are much more sensitive to temperature changes than those in other animals (1, 11). These results suggest that when human oocytes are manipulated in vitro, great care should be taken to maintain the stable temperature, especially during micromanipulation in processes such as ICSI.

### Heating Systems

Three different systems to maintain thermodynamic stability were used in our study. System 1 (Bioprotechs T.C.O. Culture Dish System; Bioprotechs Inc., Butler, PA) is a temperature controller, a stage adapter, and a dish with a specially coated clear glass bottom (0.15 mm thick). System 2 (Bioprotechs T.C.O. Culture Dish System plus Bioprotechs objective lens heater) also keeps the objective lens to the specified temperature; the objective heater surrounds the Neofluar  $\times 40$  strain-free objective lens. System 3 (Fryer A-50 heating stage; Fryer Co., Carpentersville, IL) warms the plastic Petri dish and is not thermostated. Oocytes cultured in this heating stage could not be imaged because the PolScope requires imaging through a glass-bottomed Petri dish.

Because the meiotic spindle is important for normal chromosome alignment during meiosis, the examination of the spindle may provide a useful approach to diagnosing oocyte abnormalities in women undergoing ICSI. Abnormal spindles may contribute to aneuploidy (12), disruption of embryo development and implantation, and decreased pregnancy rates, especially in older women (13–15). These results suggest that maintenance of spindle integrity should improve fertilization and subsequent embryo development after ICSI.

### Spindle Examination and ICSI With the PolScope

To maintain temperature during in vitro manipulation, various systems to ensure thermodynamic stability have been developed. However, no comparisons have been made of the levels of thermodynamic stability provided by these systems and their impact on ICSI outcome. We hypothesized that rigorous temperature control would improve embryo developmental ability and implantation and pregnancy rates. In this study, we compared the thermodynamic stability of three systems and employed the PolScope to image spindles for examining the impact of these systems on spindle integrity as well as on fertilization and clinical pregnancy.

For imaging spindles and ICSI, each oocyte was placed in a 5- $\mu$ L drop of modified HTF covered with warm paraffin oil (Gallard-Schlesinger, Coral Place, NY). Oocytes were imaged in systems 1 and 2 under a Zeiss Axiovert 100 with a Neofluar  $\times 40$  strain-free objective lens and LC-PolScope optics and controller (Cambridge Research & Instrumentation, Inc., Woburn, MA), combined with a computerized image analysis system (MetaMorph Universal Imaging System, West Chester, PA). After imaging, ICSI was performed. Because the first polar body position in many eggs did not accurately predict the spindle position, ICSI was conducted after the oocytes were rotated to place the spindle (oocytes with spindles in systems 1 and 2) or the first polar body (oocytes without spindles in systems 1 and 2 and all oocytes in system 3) at 90 degrees relative to the injection needle. Four or less than four eggs were imaged and used for ICSI each time by skilled embryologists. The time for ICSI in each group (four eggs) depended on the available number of sperm, ranging from less than 10 minutes to 30 minutes.

After ICSI, oocytes were cultured for examination of fertilization in P1 medium (Irvine Scientific) supplemented with 6% SSS.

Sixteen to 18 hours after ICSI, fertilization was evaluated. Oocytes with two pronuclei were considered normally fertilized. Fertilized eggs were washed three to four times and cultured in freshly prepared growth medium (P1 + 10% SSS) which had been equilibrated in a CO<sub>2</sub> incubator overnight. Embryo transfer was conducted on day 3, 5, or 6, depending upon laboratory protocol. After day 3, embryos were cultured in blastocyst medium (Irvine Scientific) containing 10% SSS.

## Temperature Monitor

Systems 1 and 2 allowed direct monitoring of the temperature of the medium in the dish. The temperature of the culture medium was measured by a Bioptechs temperature controller in systems 1 and 2, and also was confirmed using a thermistor (HFT-80 Digital surface thermometer, Anritsu Meter Co., Franklin Lakes, NJ). Temperature could be monitored during imaging and ICSI. Temperature of culture medium in system 3 was measured using the HFT-80 thermometer, but could not be monitored during ICSI.

## Statistical Analysis

Comparisons were conducted by  $\chi^2$  test.  $P < .05$  was considered to be statistically significant.

## RESULTS

When the unheated objective lens approached the oocytes cultured in system 1, the temperature dropped 3°C. When a conventional stage warmer (system 3) was used, the temperature of the culture medium was 4°C lower than the setting temperature. However, system 2 maintained the temperature in the medium at 37° ± 0.1°C. When oocytes were examined with the PolScope (as shown in Table 1), spindles were imaged in more oocytes ( $P < .05$ ) with system 2 (81.2%) than with system 1 (61.4%). Fertilization rates were also significantly higher ( $P < .05$ ) when oocytes were examined and used for ICSI with system 2 (78.8%) than with system 1 (56.7%). The latter was the same rate as the oocytes used for ICSI with a conventional stage warmer (64.0%). Most importantly, a significantly higher ( $P < .05$ ) clinical pregnancy rate followed ICSI performed with system 2 (51.7%) than with system 1 (25.0%) or system 3 (23.1%). No differences were observed in the average patient's age, mean number of previous cycles, or day 3 FSH level and estradiol concentration among the three groups (see Table 1).

## DISCUSSION

When advanced thermodynamic techniques are used to control oocyte temperature, thermal control is at its most rigorous; spindles are stabilized, and fertilization and clinical pregnancy rates increase after ICSI. Without an objective

**TABLE 1**

Patient information and clinical outcome.

	System 1	System 2	System 3
No. of patients	40	29	52
Average patient's age	33.8 ± 4.4	34.1 ± 4.6	34.1 ± 4.4
Average no. of cycles	2.3 ± 1.4	2.8 ± 1.2	2.6 ± 1.8
Day 3 FSH	6.1 ± 1.8	6.3 ± 2.6	6.2 ± 2.5
E <sub>2</sub> level (pre-hCG)	1346.4 ± 608.3	1344.8 ± 552.4	1417.6 ± 763.5
E <sub>2</sub> level (day for hCG)	1780.3 ± 805.1	1809.0 ± 815.6	1926.8 ± 980.8
No. of eggs examined	402	298	433
No. of eggs/patient	8.3	10.0	10.3
Eggs with spindle (%)	61.4 <sup>a</sup>	81.2 <sup>a</sup>	NA
Fertilization rate (%)	56.7 <sup>a</sup>	78.8 <sup>a</sup>	64.0 <sup>a</sup>
Pregnant rate (%)	25.0 <sup>a</sup>	51.7 <sup>a</sup>	23.1 <sup>a</sup>

<sup>a</sup>  $P < .05$  within the same row.

Note: NA = not applicable.

Wang. Spindle stabilization in human eggs. *Fertil Steril* 2002.

lens heater, the objective lens at ambient temperature cooled the egg as it was brought into focus, even when the dish itself was warmed. The objective lens acted as a heat sink, but the objective warmer overcame this effect and stabilized the oocytes' temperature. According to previous studies, microtubules can repolymerize to form spindles after transient cooling only in a small number of oocytes (1, 3). In some oocytes, even in those with recovered spindles, presumably the function of the reconstituted spindles is disrupted, because the resulting pregnancy rates were half those produced by rigorous thermal control during ICSI. Imaging with the PolScope therefore provides the ability to visualize the impact of laboratory manipulation on the structure of the meiotic spindle during ICSI.

The results obtained in the present study suggest that birefringent spindles in living human oocytes can serve as markers to monitor oocyte quality control during egg micro-manipulation. Although we do not know why temporary cooling reduces fertilization, embryo development, and pregnancy rates, presumably abnormal spindles formed during in vitro manipulation contribute to subsequent aneuploidy formation. It has been reported that the rate of aneuploidy in humans is very high (12–14), but we still do not know to what extent this tendency is exaggerated by in vitro manipulation. Because a high proportion of oocytes from older women showed abnormal spindles (5) and aneuploidies (12, 14, 15), spindles in oocytes from older women may be even more sensitive to temperature and other changes than are spindles from young women.

Recovery of spindles after rewarming was observed only in a small number of human oocytes (1, 3). Oocytes undergoing ICSI on a conventional stage warmer encountered a temperature of only 33° to 34°C, and a ×40 objective lens used for ICSI further reduced the temperature of the medium. Increasing the temperature of the conventional stage

warmer may increase the dish temperature, but also could damage some oocytes exposed to superphysiologic higher temperatures, even for a short time. Heat damage does not appear recoverable (4). Therefore, in the present study, we used an objective lens heater attached to the  $\times 40$  objective lens during oocyte examination and ICSI to maintain the medium temperature at  $37^\circ \pm 0.1^\circ\text{C}$ , and thus significantly increased the proportion of oocytes with spindles.

It was interesting that some oocytes (<20%) did not show birefringent spindles. Oocytes not exhibiting spindle birefringence most likely 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.

Whether decreased development in oocytes without spindles results in aneuploidies is not clear, but our data are consistent with such a possibility. Further experiments examining the relationship between spindle birefringence and aneuploidy are needed to test this hypothesis.

In conclusion, rigorous thermal control produced by a novel heating system stabilized spindles and increased fertilization, embryo development, and clinical pregnancy rates achieved after ICSI. The PolScope technique can be used to monitor in vitro manipulation environments by imaging spindle integrity in living oocytes.

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