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Limited recovery of meiotic spindles in living human oocytes after cooling–rewarming observed using polarized light microscopy

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BACKGROUND: Spindles are formed from microtubules and are exquisitely sensitive to changes in temperature. An orientation-independent polarized light microscope, the Polscope, can be used to image spindles in living oocytes allowing analysis of spindle kinetics in the living state. This study examined the effects of cooling on spindle disassembly in living human oocytes and spindle recovery after rewarming. METHODS: Oocytes were imaged continuously with the Polscope during cooling and rewarming. The quantity of microtubules in the spindles was measured by its birefringence using the Polscope. RESULTS: Spindles had completely disassembled by 5 min after cooling and recovered by 20 min after rewarming to 37°C if rewarming started soon after the oocyte's temperature dropped to room temperature. However, when oocytes were cooled and kept at 33, 28 or 25°C for 10 min and then warmed, it was found that warming allowed 5/5, 2/5 and 0/5 oocytes of the spindles to recover respectively. CONCLUSIONS: These results indicate that human meiotic spindles are exquisitely sensitive to alterations in temperature. The maintenance of temperature at 37°C during in-vitro manipulation is important for spindle integrity and, therefore, is likely to be important for normal fertilization and subsequent embryo development.

Key words: Cooling/human oocytes/polarization microscope/spindle

Introduction

Meiotic spindles are composed of microtubules and are important for chromosome alignment and separation of maternal chromosomes during fertilization. The meiotic spindles of most mammals are very sensitive to fluctuations in temperature (Moor and Crosby, 1985; Pickering and Johnson, 1987; Aman and Parks, 1994) including human (Pickering *et al.*, 1990; Almeida and Bolton, 1995). This finding is important for assisted human reproductive technology, such as IVF and intracytoplasmic sperm injection (ICSI), since oocytes are exposed to in-vitro conditions during the procedure. Temperature fluctuations during in-vitro manipulation may disrupt the spindles (Aman and Parks, 1994; Almeida and Bolton, 1995) and contribute to abnormal chromosome distribution. Insemination of such oocytes may cause failed fertilization or abnormal fertilization, such as aneuploidy. Most of our knowledge about spindle dynamics is obtained from studies of fixed samples imaged by immuno-fluorescence microscopy or electron microscopy (Moor and Crosby, 1985; Pickering and Johnson, 1987; Pickering *et al.*, 1990; Aman and Parks, 1994; Almeida and Bolton, 1995). While these methods provide high resolution images, they are static and therefore do not allow study of the dynamic behaviour of spindles in individual oocytes. Moreover, the images cannot be compared in the same spindles. Recently, in our laboratory, we successfully applied an orientation-independent polarized light microscope, the Polscope, to study spindle architecture in living mammalian oocytes (Silva *et al.*, 1999; Liu *et al.*, 2000a,b) including human oocytes (Wang *et al.*, 2001a,b). Exposure of mammalian oocytes or embryos to the Polscope is non-invasive (Liu *et al.*, 2000a), so the Polscope has the potential to be used in human IVF clinics (Wang *et al.*, 2001a). The other advantage of the Polscope is that spindles can be imaged continuously in the same oocytes to study the architectural dynamics of the same spindles. In the present study, using the Polscope, we examined the dynamics of human meiotic spindles during changes in temperature.

Materials and methods

Source 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 scollected 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 cumulus-oocyte complexes in modified human tubule fluid (HTF) (Irvine Scientific) containing 80 IU/ml hyaluronidase (Sigma Chemical Co., St Louis, MO, USA). Oocytes that released the first polar body were used for ICSI and oocytes without a first polar body were cultured *in vitro*. In-vitro maturation was conducted in P1 medium supplemented with 6% SSS at 37°C, 5% CO_2 in air with 100% humidity. At 22–24 h after culture, oocytes that released the first polar body were used in the study. The patients were informed that the immature oocytes were not used for ICSI and would not be inseminated even after nuclear maturation after culture as there were potential chromosome abnormalities in these oocytes. Therefore, after cooling and rewarming, all oocytes were not used for insemination and were discarded according to hospital policy and laboratory protocols.

Spindle examination in living oocytes with the Polscope

For imaging spindles, each oocyte was placed in a 5 µl drop of HEPES-buffered HTF covered with warm paraffin oil (Gallard-Schleserger, Coral Place, NY, USA) in a Bioptechs Delta T.C.O. Culture System (Bioptechs Inc., Butler, PA, USA). The system comprises a temperature controller, a stage adapter and the T.C.O. dish that has a specially coated clear glass (0.15 mm thick) bottom. The temperature of dishes was maintained and monitored to ± 0.1°C throughout the study. Oocytes were examined under a Zeiss Axiovert 100 microscope with a Neofluar 40× strain-free objective equipped with the LC Polscope (Cambridge Research and Instrumentation, Woburn, MA, USA), combined with a computerized image analysis system (MetaMorph Universal Imaging System, West Chester, PA, USA).

Experimental Designs

Experiment 1

Oocytes were cooled from 37°C to room temperature (25–26°C) after turning off the temperature controller. Oocytes were imaged every 30 sec and the temperature was recorded for each image. Soon after the temperature reached room temperature, the temperature controller was turned on and spindles were imaged every 30 sec until 30 min after the stage temperature reached 37°C. Spindle retardance, a measure of microtubule density, was determined with the Metamorph computer image system.

Experiment 2

Oocytes were cooled from 37 to 33, 28 and 25°C, maintained at the desired temperature for 10 min and then rewarmed to 37°C. In order to avoid the influence of prolonged exposure of oocytes to light, oocytes were imaged before cooling, 5 and 10 min after cooling, and 10 and 20 min after rewarming to 37°C.

Results

Experiment 1

Figure 1 shows the medium temperature during cooling and rewarming of human oocytes in culture. When the controller was turned off, the temperature dropped rapidly during the first 3 min, then dropped more gradually to reach room temperature. After turning the temperature controller back on, the temperature reached 37°C in 3.5 min. Spindles in the same individual oocytes were imaged every 30 sec with the Polscope. As shown in Figure 2, at 37°C (before cooling) intact spindles were observed in oocytes (Figure 2A) with a maximum retardance of 5.78 nm. At 1.5 min after cooling, the medium's temperature dropped to 31.9°C and the spindle began to disassemble (Figure 1B), with retardance first dropping to 4.56 nm. Spindles completely disassembled 5 min after the start of cooling (Figure 2C), with a maximum retardance of 2.44 nm, when the temperature was 27.1°C. After rewarming, the spindles started to reassemble (Figure 2D) 3 min after the temperature reached 37°C (6.5 min after turning on the temperature controller), with a maximum retardance of 4.07 nm. The retardance (4.09 nm) did not increase at 10 min after the temperature reached 37°C (Figure 2E). At 20 min after warming to 37°C, spindles had recovered with a maximum retardance of 5.01 nm (Figure 2F). Spindle disassembly was accompanied by shrinkage of the oocyte (Figure 2C), but warming completely restored oocyte volume (Figure 2D–F). Also we observed that the apparent random motion of organelles and yolk granules in the oocyte was markedly reduced at room temperature compared with 37°C.

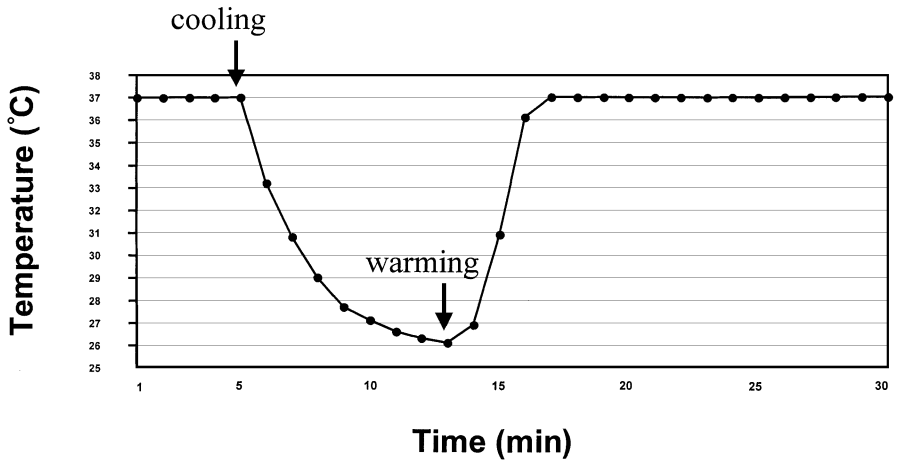

Figure 1. Medium temperature during cooling and rewarming of human oocytes. Temperature was recorded every 30 sec and spindle was imaged at each temperature point.

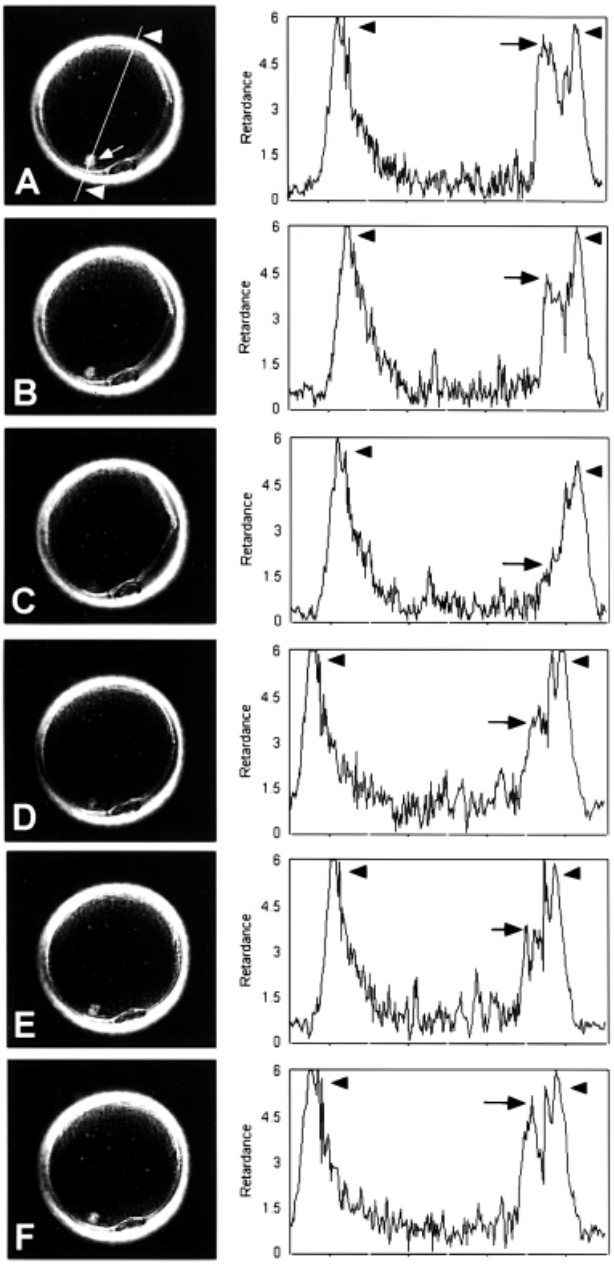

Figure 2. Spindle dynamics after cooling and rewarming in a human oocyte. Oocyte was imaged at 37°C, then cooled to room temperature and finally rewarmed to 37°C. The graphs on the right column indicate retardance (nm), a measurement of microtubule density at the location of the spindle (arrow). Zona birefringence (arrowhead) can also be seen. Images were obtained at 37°C (**A**), 31.9°C, 1.5 min after cooling (**B**), and 27.1°C, 5 min after cooling (**C**). The images in (**D**–**F**) were obtained after rewarming to 37°C for 3 min (**D**), 10 min (**E**) and 20 min (**F**). Original magnification, ×120.

Experiment 2

As shown in Figure 3, when oocytes were cooled to 33, 28 and 25°C and kept at the desired temperature for 10 min, spindles in oocytes had disassembled completely by 5 min after cooling to 25 and 28°C or by 10 min after cooling to 33°C. Warming to 37°C for 10 min allowed 5 out of 5 oocytes that had cooled to 33°C to recover their spindles. However, only 2 out of 5 oocytes which had been cooled to 28°C and no oocytes (0/5) which had been cooled to 25°C recovered their spindles by 20 min after rewarming. Spindle images did not change in oocytes that had been kept at 37°C during 40 min of examination.

Discussion

The meiotic spindles are crucial for normal chromosome alignment and separation of chromosomes during meiosis, and for normal fertilization. However, experimental evidence indicates that meiotic spindles are exquisitely sensitive to environmental changes, especially fluctuations in temperature (Moor and Crosby, 1985; Pickering and Johnson, 1987; Pickering *et al.*, 1990; Aman and Parks, 1994; Almeida and Bolton, 1995). Disruption of the spindle increases aneuploid formation, which is one of the most observed patterns of abnormal fertilization in the human. When oocytes are manipulated *in vitro*, such as for ICSI or IVF, fluctuations in temperature inevitably occur, even in the most careful hands. Thus disruption of spindle integrity could be expected. This may be one of the reasons that ~30% of oocytes used for ICSI are not fertilized, or fertilize abnormally. Meiotic spindles in human oocytes are disrupted even by slight fluctuations in temperature, and their recovery after warming is limited (Pickering *et al.*, 1990; Almeida and Bolton, 1995). Chromosomes were abnormally distributed in spindles, even when spindles partially recovered after rewarming (Moor and Crosby, 1985; Pickering and Johnson, 1987; Aman and Parks, 1994). Recently, Zenzes *et al.* also reported that spindles in human oocytes disappeared after cooling to 0°C (Zenzes *et al.,* 2001). However, they found that the chromosomes did not disperse after cooling (Zenzes *et al.,* 2001). This may be because chromosomes are held by spindle fibres and chromosome separation is controlled by spindle dynamics. It is possible that rapid disassembly of spindles induced by cooling does not induce chromosome dispersion in the cytoplasm, however the fact remains that oocytes cannot undergo meiosis without spindles. Even though oocytes can partially recover their spindles after rewarming, abnormal meiosis may occur if these oocytes are fertilized. Although Zenzes *et al.* examined spindle morphology after cooling to 0°C (Zenzes *et al.,* 2001), they did not examine their recovery after rewarming or the relationship between spindles and chromosomes. In the present study, spindles did not completely recover when the oocytes were cooled to room temperature for more than 10 min, and we would therefore expect even less recovery following cooling to 0°C. It is necessary to use animal oocytes to examine the relationship between spindle recovery and meiosis after cooling-rewarming procedures.

In our previous studies, we found that the presence or absence of a birefringent spindle imaged with the Polscope in human oocytes before ICSI, significantly affected subsequent fertilization (Wang *et al.*, 2001a) and embryonic development (Wang *et al.*, 2001b). Lower fertilization and blastocyst formation rates were also observed in oocytes without spindles compared with oocytes with spindles (Wang *et al.*, 2001a,b).

Our previous studies indicated that only about 60–80% of

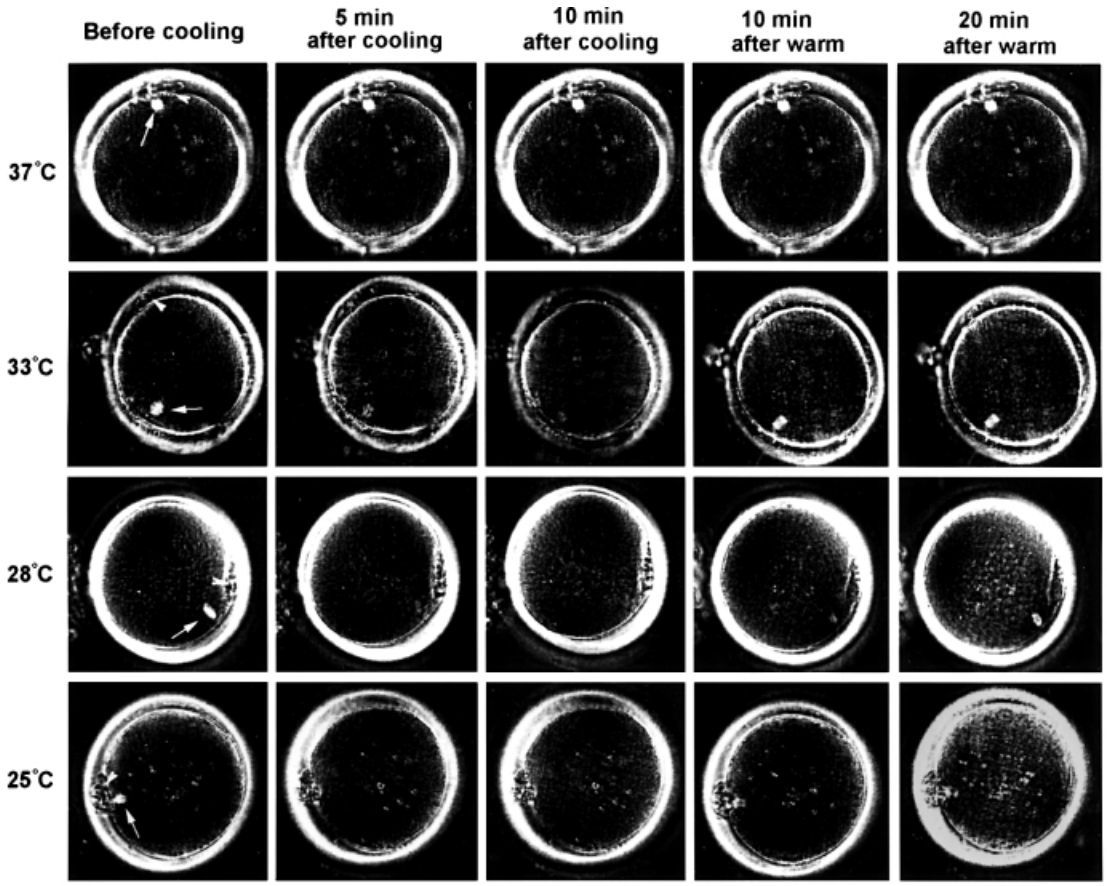

Figure 3. Spindle images in human oocytes after cooling and rewarming. The oocytes were imaged after being maintained at 37°C or cooled to 33, 28 and 25°C and then rewarmed to 37°C. Images were taken before cooling, 5 and 10 min after cooling and again 10 and 20 min after rewarming to 37°C. Arrows indicate the spindles, and arrowheads indicate the first polar body. Original magnification, ×120.

oocytes at metaphase II exhibit spindle birefringence imaged with the Polscope, even when we used a rigorous thermal control during imaging (Wang *et al.*, 2001a). The present results indicate that spindles in human oocytes are depolymerized even after a slight decline in temperature. These results suggest that even the transient movement of human oocytes required by IVF or ICSI may contribute to spindle disassembly, and that recovery after rewarming is limited to a proportion of oocytes, depending on the temperature decrease.

Previous studies have examined changes in spindles after the temperature was reduced to room temperature (Pickering and Johnson, 1987; Pickering *et al.*, 1990; Almeida and Bolton, 1995) or to 0°C (Zenzes *et al.,* 2001). The present study examined in more detail the effects of even more subtle temperature changes. The results add new information to our knowledge about the relationship between temperature changes and spindle dynamics. We found that a reduction to just 33°C resulted in the depolymerization of spindles within 10 min. Moreover, the lower the temperature, the more rapidly the spindles depolymerized. Recovery of spindles cooled to 33°C was observed in all oocytes after rewarming, but fewer oocytes recovered spindles after they were cooled to 28°C and none recovered after cooling to room temperature. However, as shown in Experiment 1, if oocytes were rewarmed soon after the temperature dropped to room temperature, oocytes could recover their spindles, although not completely so. These results indicate that spindle disassembly is temperature- and time-dependent. It is important to manipulate oocytes carefully and rapidly under in-vitro conditions.

In this study, we used spindle retardance to measure microtubule density in the spindles, which has been discussed previously (Oldenbourg, 1996, 1999; Liu *et al.*, 2000b). We found that even if spindles recovered after rewarming, the density of microtubules was decreased, suggesting that the spindles had not re-polymerized completely. During the process of microtubule depolymerization and re-polymerization, it is possible that the relationship between microtubules and chromosomes also changed. Such changes, even subtle, might be expected to contribute to aneuploidy after fertilization. Further study is needed to clarify this possibility. Furthermore, the results of this study build upon proven studies of the temperature effect on meiotic spindles by demonstrating the value of the Polscope in studying the physiological activities of spindles. Such an approach cannot be employed in fixed oocytes. Most importantly, the data were obtained and compared in the same individual oocytes thus avoiding the variances between oocytes, allowing us to conduct measurements even with the few human oocytes available for the study.

Spindle architecture is changed in aged (Eichenlaub-Ritter, 1988; Wang *et al.*, 2001a) human oocytes and in oocytes from

older women (Battaglia *et al.*, 1996; Volarcik *et al.*, 1998). Such age-related changes also may contribute to aneuploid formation. At this time we do not know whether advanced maternal age increases the spindle's sensitivity to temperature. Aneuploidy is one of the most commonly observed patterns of abnormal fertilization in the human and the dramatic drop in the pregnancy rate in older women is closely related to the occurrence of aneuploidies (Benadiva *et al.*, 1996; Munné, 1999). If a relationship between spindle retardance and chromosome distribution, as well as to subsequent fertilization could be determined, the Polscope technology may be useful in human IVF clinics to help diagnose aneuploidy.

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