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Overheating is detrimental to meiotic spindles within *in vitro* matured human oocytes

Xiao-Fang Sun[1], *Wei-Hua Wang*[2] *and David L. Keefe*[3]
Guang-Zhou Second Hospital, Gung-Dong, China; Tomball Regional Hospital, Tomball, Texas, USA; and Women and Infants Hospital of Rhode Island, Providence, Rhode Island, USA

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Summary

The present study was designed to examine the effects of overheating on meiotic spindle morphology within *in vitro* matured human oocytes using a polarized light microscope (Polscope). Immature human oocytes at either germinal vesicle or metaphase I stage were cultured *in vitro* for 24–36 h until they reached metaphase II (M-II) stage. After maturation, oocytes at M-II stage were imaged in the living state with the Polscope at 37, 38, 39 and 40 °C for up to 20 min. After heating, oocytes were returned to 37 °C and then imaged for another 20 min at 37 °C. The microtubules in the spindles were quantified by their maximum retardance, which represents the amount of microtubules. Spindles were intact at 37 °C during 40 min of examination and their maximum retardance (1.72–1.79) did not change significantly during imaging. More microtubules were formed in the spindles heated to 38 °C and the maximum retardance was increased from 1.77 before heating to 1.95 at 20 min after heating. By contrast, spindles started to disassemble when the temperature was increased to 39 °C for 10 min (maximum retardance was reduced from 1.76 to 1.65) or 40 °C for 1 min (maximum retardance was reduced from 1.75 to 1.5). At the end of heating (20 min), fewer microtubules were present in the spindles and the maximum retardance was reduced to 0.8 and 0.78 in the oocytes heated to 39 °C and 40 °C, respectively. Heating to 40 °C also induced spindles to relocate in the cytoplasm in some oocytes. After the temperature was returned to 37 °C, microtubules were repolymerized to form spindles, but the spindles were not reconstituted completely compared with the spindles imaged before heating. These results indicate that spindles in human eggs are sensitive to high temperature. Moreover, maintenance of an *in vitro* manipulation temperature of 37 °C is crucial for normal spindle morphology.

Keywords: Heating, Human oocytes, Meiotic spindle, Polscope

Introduction

The initiation of most mammalian oocyte maturation is indicated by breakdown of the germinal vesicle nucleus in immature oocytes. As the oocyte progresses into metaphase I, a meiotic spindle is formed and the chromosomes are aligned at the equator of the spindle. After that, the first polar body with a set of chromosomes is released from the oocytes, the oocytes progress to metaphase II stage (M-II) and a meiotic spindle is formed. Chromosomes are aligned at the equator of this spindle until fertilization, which triggers segregation of chromosomes and induces the oocyte to release the second polar body and reduce the chromosome number to haploid. Meiotic spindle and spindle checkpoint proteins are responsible for normal chromosome alignment and separation during meiosis. Accurate chromosome segregation relies on the attachment and alignment of chromosomes on the meiotic spindle. Mistakes in these processes result in aneuploid formation. An abnormal spindle may lead to misalignment of chromosomes (Battaglia *et al*.,

All correspondence to: Wei-Hua Wang, 13414 Medical Complex Dr, Suite 7, Tomball TX 77375, USA. Tel: +1 (281) 3571881. Fax: +1 (281) 3571865. e-mail: wangweihua11@yahoo.com
[1] Institute of Obstetrics and Gynecology, Guang-Zhou Second Hospital, Gung-Dong, China.
[2] Houston Fertility Institute, Tomball Regional Hospital, Tomball, Texas, USA.
[3] Women and Infants Hospital of Rhode Island, Providence, Rhode Island, USA.

1996), thus inducing aneuploid formation. Aneuploidy is occasionally observed in human embryos produced by *in vitro* fertilization (IVF) (Verlynsky *et al*., 2001).

It has been found that meiotic spindles are sensitive to temperature fluctuations, especially cooling, in most mammals (Moor & Crosby, 1985; Aman & Parks, 1994; Liu *et al*., 2003) including human (Pickering *et al*., 1990; Wang *et al*., 2001*b*). All findings indicated that cooling could induce microtubule disassembly while warming of the oocytes could re-polymerize the microtubules to recover the spindle in some cases. However, only limited recovery was observed in most cases, depending on the treatment (Moor & Crosby, 1985; Pickering *et al*., 1990; Aman & Parks, 1994; Wang *et al*., 2001*b*; Liu *et al*., 2003). As the temperature generally drops during *in vitro* manipulation of oocytes, oocytes are usually maintained at approximately 37 °C during *in vitro* manipulation by using various kinds of temperature controllers, such as heating plates. However, these heating systems are not stable, and fluctuations in the temperature (in either direction) can occur. It is well known that a lower temperature can induce spindle disassembly, but little is known about the effect of overheating.

It has been reported that heating bovine oocytes did not significantly affect oocyte morphology and their subsequent fertilization and embryo development (Ju *et al*., 1999; Kawarsky & King, 2001). However, it has been found that mouse oocytes are very sensitive to heating treatment (Komar & Kujawa, 1985; Hendrey & Kola, 1991). It would appear that species-specific differences exist among animals. Previous studies have indicated that human oocytes are very sensitive to cooling, and even transient cooling to room temperature or a slight lower temperature than body temperature induces irreversible disruption of the meiotic spindle in human oocytes (Pickering *et al*., 1990; Wang *et al*., 2001*b*). It is therefore possible that human oocytes are also sensitive to increases in temperature. In the present study experiments were designed to examine this possibility.

In a previous study, we used a polarization light microscope, the LC-Polscope (Wang *et al*., 2001*a*), which is a technique that relies on a material's optical properties to examine microstructure within cells (Inoue, 1953; Oldenbourg, 1999), to examine meiotic spindles in living human oocytes. The advantage of spindle imaging with the Polscope is that the spindle can be imaged in the same oocyte in the living state, thus time-lapse changes in the same spindle can be observed during treatment. This technology can be used to study spindle dynamics more precisely and it is not necessary to use many oocytes or to use fixative and specific antibodies to stain the spindles in the oocytes. This is particularly important in the human, as limited numbers of oocytes are available for research purpose. In the present study, the spindle changes after heating treatment were imaged with the Polscope in living human oocytes.

Materials and methods

Source of oocytes

Approval was obtained from our hospital institutional review committee to study immature human oocytes and to study images of oocytes obtained during human IVF. Oocytes were collected from stimulated ovaries of consenting patients undergoing oocyte retrieval for intracytoplasmic sperm injection (ICSI). After retrieval, oocytes were cultured in P1 medium (Irvine Scientific, Santa Ana, CA) 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 HTF (Irvine Scientific) containing 80 IU/ml hyaluronidase (Sigma Chemical, St Louis, MO). Oocytes that released the first polar body were used for ICSI and oocytes without a first polar body or oocytes at germinal vesicle stage 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. Twenty-four to thirty-six hours after culture, oocytes that released the first polar body were used in the study. The patients were informed that the immature eggs were not used for ICSI and would not be inseminated even after nuclear maturation as there were potential chromosome abnormalities in these oocytes. Therefore, after treatment no oocytes were used for insemination and all 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) in a Bioptechs Delta TCO Culture System (Bioptechs, Butler, PA). The system is comprised of a temperature controller, a stage adapter and the TCO dish that has a specially coated clear glass bottom (0.15 mm thick). The temperature of dishes was maintained and monitored to ±0.1 °C throughout the study. Oocytes were examined under a Zeiss Axiovert 100 with a Neofluar ×40 strainfree objective equipped with the LC Polscope (Cambridge Research and Instrumentation, Woburn, MA), combined with a computerized image analysis system (MetaMorph Universal Imaging System, West Chester, PA).

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Experimental design

Only oocytes that had released the first polar body and also showed birefringent spindles when imaged with the Polscope at 37 °C were used in the study. We found that about 75% of *in vitro* matured human oocytes showed birefringent spindles with the Polscope (Wang & Keefe, 2002). For heating treatment, oocytes with birefringent spindles were exposed to shifts in temperature at 37 °C (control), 38 °C, 39 °C and 40 °C. The temperature of microdrops was also monitored and confirmed using a thermistor (HFT-80 Digital surface thermometer, Anritsu Meter, Franklin Lakes, NJ). Spindles then were imaged continuously for 20 min. Thereafter, oocytes were returned to 37 °C and imaged for another 20 min to examine spindle recovery. Images were taken every 60 s during the treatment, which was programmed by the computer software. Only one oocyte was imaged at a time at the experimental temperature. Ten replications (10 different oocytes) were conducted for each temperature treatment.

Statistical analysis

Retardance is the primary quantity measured with the compensator in the Polscope. The measured peak (or maximum) retardance of microtubules is directly proportional to the number of microtubules in the spindle (Oldenbourg, 1999). Therefore, in the present study, maximum retardance of microtubules was measured with the computer software and the data (mean ± SD) analysed by ANOVA. A probability of $p < 0.05$ was considered to be statistically significant.

Results

As shown in Fig. 1, spindle retardance can be measured longitudinally and vertically. However, vertical measurement (Fig. 1A) can be used to quantify more microtubule bundles in the spindle than longitudinal measurement (Fig. 1B), which may measure only one microtubule. Therefore, in the present study we used the vertical measurement to quantify the microtubules.

As shown in Fig. 2, spindles were intact at 37 °C during 40 min of examination in all oocytes examined and the maximum microtubule retardance was 1.72 ± 0.05 to 1.79 ± 0.1 nm (Fig. 3). When oocytes were heated to 38 °C, more microtubules were formed in the spindles during heating, and the maximum retardance was increased from 1.76 ± 0.08 before heating to 1.92 ± 0.15 nm at 20 min after heating, but no statistical difference was observed. The images and maximum retardance were the same after the temperature was returned to 37 °C compared with the values before heating (1.76 ± 0.09 nm). However, spindles started to disassemble when the temperature was increased

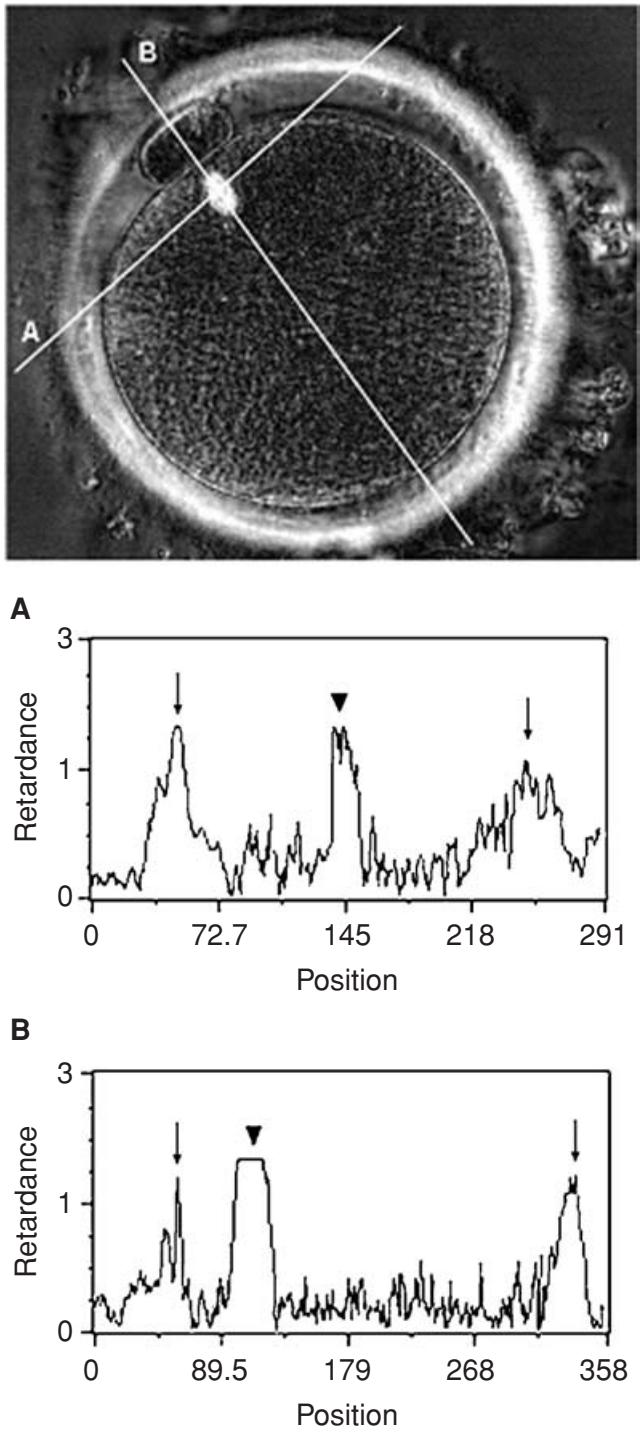

Figure 1 Meiotic spindle imaged in a living human oocyte with the Polscope and its retardance measurements. (A) Retardance measured in the spindle vertical direction, see line A in the oocyte image; (B) retardance measured in the spindle longitudinal direction, see line B in the oocyte image. Arrows indicate zona birefringent retardance and arrowheads indicate spindle birefringent retardance.

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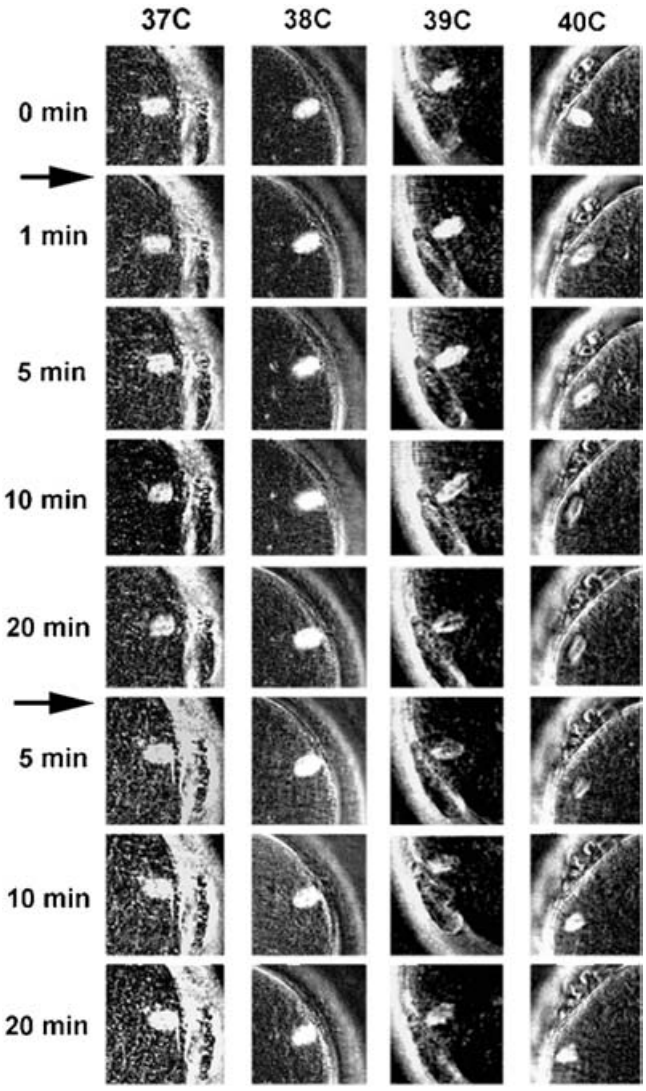

Figure 2 Spindle images imaged with the Polscope in living human oocytes after overheating. The oocytes were imaged at 37 °C or imaged after being heated to 38, 39 and 40 °C, and then imaged after being returned 37 °C. Images were taken before heating (37 °C), 1, 5, 10 and 20 min after heating, and again 5, 10 and 20 min after returning the oocytes to 37 °C. Top arrow indicates the start of heating and the bottom arrow indicates cessation of heating. Scale bar represents 12 μm.

to 39 °C. The maximum retardance was decreased ($p < 0.01$) to 0.8 ± 0.09 nm at 20 min after heating. When the oocytes were returned to 37 °C, a recovered spindle was observed (Fig. 2). Although the retardance (1.67 ± 0.08 nm) was not significantly different from the values before heating, the morphology of the recovered spindle was completely different from the original image. It was found that heating to 40 °C induced a faster (1 min) partial disassembly of the spindle and the maximum retardance was significantly ($p < 0.05$) reduced (from 1.75 ± 0.12 nm to 1.5 ± 0.07 nm). Fewer microtubules were found in the spindles at the end of heating (20 min) and the maximum retardance was 0.78 ± 0.08 nm. Heating to 40 °C also induced spindles to relocate (6 out of 10 eggs) in the cytoplasm; this occurred during the first minute of heating. After the temperature was returned to 37 °C, some microtubules were repolymerized to form spindles after about 10 min of culture. Although the maximum retardance was increased after 20 min of culture, the morphology of the recovered spindle was obviously different from the original images, indicating incomplete spindle recovery (Fig. 2).

Discussion

It has been found that the meiotic spindles of most mammals (Moor & Crosby, 1985; Aman & Parks, 1994; Almeida & Bolton, 1995; Liu *et al*., 2003), including human (Pickering *et al*., 1990; Wang *et al*., 2001*b*), are very sensitive to a decline in temperature. It would appear that spindles in human oocytes are more sensitive to a temperature decline than those in other animals, as spindles disassemble completely when the temperature is lowered to 33 °C, a 4 °C difference from body temperature (Wang *et al*., 2001*b*). The present finding indicates that meiotic spindles in human oocytes are also sensitive to the rise in temperature, and abnormal spindles were observed when the temperature was increased to 39 °C, a 2 °C difference from body temperature. These results may suggest that spindles in human oocytes are more sensitive to higher temperature than lower temperature. Such a finding is important for assisted human reproductive technology, since the egg temperature may decrease or increase during *in vitro* manipulation. Although oocytes have the ability to recover spindles after the temperature is returned 37 °C, limited and incomplete recovery is observed (Wang *et al*., 2001*b*; present study). Therefore, during spindle recovery chromosomes may be rearranged on the spindle and mistakes in the segregation between microtubules and chromosomes may occur. This may be one of many unknown factors that induce aneuploidy in human embryos (Verlynsky *et al*., 2001; Munné, 2002).

Many studies have been conducted to examine the effects of low temperature on meiotic spindle dynamics, as *in vitro* (room) temperature is lower than body temperature in most cases (Moor & Crosby, 1985; Pickering *et al*., 1990; Aman & Parks, 1994; Wang *et al*., 2001*b*; Liu *et al*., 2003). However, little attention has been paid to higher temperature. Previous studies in bovine oocytes indicated that heating oocytes at M-II stage to 40.5–43 °C did not affect oocyte

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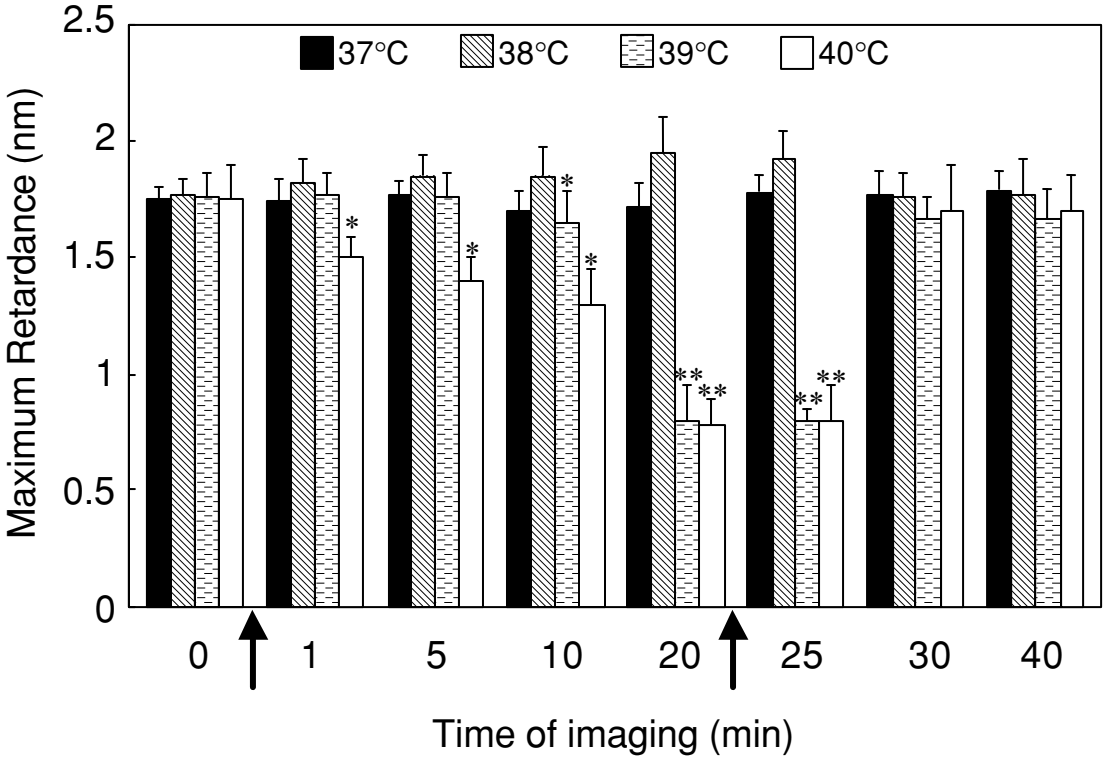

Figure 3 Maximum retardance of microtubules in meiotic spindles of *in vitro* matured human oocytes during heating. The oocytes were imaged after being maintained at 37 °C (control) or after being heated to 38, 39 and 40 °C, and then imaged again after being returned to 37 °C. Images were taken before heating (37 °C), 1, 5, 10 and 20 min after heating and then 5, 10 and 20 min after returning to 37 °C. The first (left) arrow indicates the start of heating and the second (right) arrow indicates the cessation of heating and a return to 37 °C. Data are expressed as the mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$.

morphology (Kawarsky & King, 2001) and subsequent fertilization and early embryo development (Ju *et al*., 1999). However, mouse oocytes were sensitive to high temperature (Komar & Kujawa, 1985; Hendrey & Kola, 1991). It would appear that species-specific differences exist among animals. It is probable that the thermolability of oocytes is related to the presence of heat shock proteins in the oocytes (Komar & Kujawa, 1985; Hendrey & Kola, 1991; Ju *et al*., 1999; Kawarsky & King, 2001); whether such proteins are present in human oocytes is unknown.

Higher temperatures may occur during the following situations. (1) Ovarian stimulation for egg retrieval. The body temperature of the patient may rise during stimulation by a physiological reaction or a pathological reaction such as fever. (2) Egg retrieval and *in vitro* manipulation of oocytes. Electronic and artificial errors in the heating system may occur during these procedures, thus the temperature of the heating system may be higher than expected, which in turn raises the egg temperature. Hence, overheating may occur in human oocytes during infertility treatment. Recently, it has been found that the percentage of aneuploid embryos in women undergoing IVF treatment is surprisingly high (Verlynsky *et al*., 2001; Munné, 2002). Many reasons, such as patient age (Battaglia *et al*., 1996; Verlynsky *et al*., 2001), oocyte age (Almeida & Bolton, 1995) and factors related to *in vitro* manipulation of oocytes, such as transient cooling (Pickering *et al*., 1990), have been suggested. In the present study, we found that a very short period of overheating damaged the spindle. Insemination of such oocytes may cause failed fertilization or abnormal fertilization. Such possibilities remain to be investigated in further studies.

A very interesting finding reported recently by Hunter *et al*. (1997) indicated that under *in vivo* conditions the temperature in porcine ovarian follicles 7–10 mm in diameter was always lower than that of the ovarian stroma or body temperature such as deep rectal temperature. Similar phenomena have also been reported in the rabbit (Grinsted *et al*., 1980). These studies suggest that follicular temperature could affect meiosis and oocyte cytoplasmic maturation. However, little is known about temperature effects on spindle dynamics. Although such findings have not been confirmed by *in vitro* studies, it is possible that strict temperature control for normal physiological activity is present in mammalian oocytes, and that this controlled

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temperature is important for normal function of the spindle or other factors, so as to ensure the production of normal embryos.

In summary, the present results indicate that spindles in human eggs are extremely sensitive to slight increases in temperature. Although such a situation rarely occurs during *in vitro* manipulation of human oocytes for infertility treatment, it is necessary to keep in mind that a temperature increase may damage oocytes in some cases. The present study, by using imaging of living spindles, provides more precise and detailed information for the study of spindle dynamics. Animal models, if they can mimic the human situation closely, may be necessary to study in more detail aneuploid formation in overheated or cooled oocytes.

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