

# Oocyte meiotic-stage-specific differences in spindle depolymerization in response to temperature changes monitored with polarized field microscopy and immunocytochemistry

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**Objective:** To compare the polymerization status of mouse oocyte spindles exposed to various temperatures at various stages of meiosis.

**Design:** Experimental animal study.

**Setting:** University animal laboratory.

**Animal(s):** CF1 mice.

**Intervention(s):** Immature oocytes matured to metaphase I (MI), telophase I (TI), and metaphase II (MII) were incubated at 37°C (control), room temperature (RT), or 4°C for 0, 10, 30, and 60 minutes. Spindle analysis subsequently was performed using polarized field microscopy and immunocytochemistry. Spindles of TI and MII oocytes that underwent vitrification and warming were analyzed also by immunocytochemistry.

**Main Outcome Measure(s):** Detection of polymerized meiotic spindles.

**Result(s):** At RT, and after 60 minutes at 4°C, a significant time-dependent decrease in the percentage of polymerized meiotic spindles was observed in MI and MII oocytes, but not in TI oocytes. The polymerization of TI spindles at 4°C was similar to that of TI spindles at 4°C that underwent vitrification and warming.

**Conclusion(s):** Significant differences in the microtubule dynamics of MI, TI, and MII oocytes incubated at different temperatures were observed. In particular, meiotic spindles in TI oocytes exhibited less depolymerization than did metaphase spindles. (*Fertil Steril*® 2012;97:714–9. ©2012 by American Society for Reproductive Medicine.)

**Key Words:** Oocyte, spindle, meiotic stage, temperature, depolymerization

**D**uring oocyte meiosis and maturation, the spindle controls chromosomal movement through the different stages of meiosis, while also mediating various functions essential for fertilization and early postfertilization events (1–3). The meiotic spindle is

located at the oocyte periphery, with one pole attached to the cell cortex (1, 4). As oocyte meiosis progresses and the germinal vesicle breaks down, the metaphase I (MI) spindle forms and persists as a functional intracellular structure during anaphase I (AI) and

telophase I (TI). The spindles, which persist during the MI to MII transition, present as a dynamic bipolar structure constructed of microtubules. The  $\alpha$ -tubulin and  $\beta$ -tubulin dimers that make up the spindle microtubules continuously undergo a dynamic equilibrium between free and polymerized states (1).

Currently, little is known about the effects of temperature variation on meiotic spindle dynamics in different oocyte meiotic stages. In mammals, meiotic spindles have been reported to be highly sensitive to fluctuations in temperature (2), with the microtubules that constitute the

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meiotic spindle undergoing rapid depolymerization when they are exposed to conditions even a few degrees below physiological temperature. In 1987, Pickering and Johnson first described mouse spindle depolymerization that occurred at temperatures in the range of 4°C–37°C (4). Similarly, Wang and coworkers (5) demonstrated that human oocyte spindles undergo depolymerization between 27.1°C and 31.9°C, and that cytoplasmic organelles and granules in oocytes show reduced movement at room temperature (RT). Although spindle repolymerization can occur (3), disassembly of a meiotic spindle has the potential to negatively impact the fidelity of meiotic chromatin segregation and separation (6). As a result, subsequent chromosome abnormalities can occur, including aneuploidy (3). Indeed, Shen et al. (7) have suggested that abnormal spindle morphology may be a promising predictor of aneuploidy. Spindle disorganization also can disrupt the sequence of events that lead to completion of meiosis and fertilization (8–10) and thus may contribute to the low pregnancy rates associated with some oocyte cryopreservation techniques (11, 12). In addition, several reports have detailed the disappearance and reappearance of the meiotic spindle during MII for both mouse and human oocytes that have undergone slow-rate freezing and vitrification (13–21). According to a recent study, the reappearance of the meiotic spindle in cooled or cryopreserved oocytes may occur immediately after warming (14); however, a time-dependent process also has been reported, with spindle recovery occurring 1–3 hours after thawing (3). It also has been proposed that temperature-induced oocyte microtubule depolymerization may be dependent on the nuclear maturation state of oocytes (22). The properties associated with each meiotic stage include permeability of the cytoplasmic membrane, the presence or absence of a nuclear membrane, the presence or absence of a spindle, cell size, and chromosome configuration (1, 22, 23). Besides the direct role that meiotic spindles have in meiotic progression and chromosome alignment and segregation, it would not be surprising that oocyte spindles also could be responsible for differences in temperature susceptibility related to meiotic stage.

Therefore, we hypothesize that there are temperature-dependent differences in the depolymerization/repolymerization equilibrium of oocyte spindles according to meiotic stage. Furthermore, this temperature dependence potentially could influence the response of oocytes to cryopreservation. Thus, the objective of this study was to investigate the influence of temperature, cryopreservation, and oocyte meiotic stage on spindle depolymerization and repolymerization as monitored by polarized field microscopy (PFM) and immunocytochemistry (ICC).

## MATERIALS AND METHODS

All procedures described were reviewed and approved by the Institutional Review Board (IRB) and the University Committee on Use and Care of Animals at the University of Michigan and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

## Oocyte Collection and Preparation

Five-week-old female CF1 mice (Harlan; Indianapolis, IN) received five international units (IU) of a pregnant mare's serum gonadotropin (PMSG) injected intraperitoneally to induce superovulation. Forty-eight hours later, immature oocytes with cumulus were collected from the ovaries and placed in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered Human Tubal Fluid medium (HTFH; Irvine Scientific, Santa Ana, CA) containing 10% bovine serum albumin. Cumulus cell removal was performed as described elsewhere (2) and cumulus-denuded germinal vesicle oocytes were then incubated in HTFH containing 10% of bovine serum albumin for various incubation times to MI (7 hours), TI (10 hours), and MII (14 hours). Oocytes at each phase then were assigned randomly and placed into drops of HTFH at 37°C (control), RT, or 4°C for 0, 10, 30, and 60 minutes before the polymerization status of the spindles was determined using PFM or ICC. In all experiments, RT was 22°C.

## PFM

Oocytes were examined using an inverted microscope (Olympus IX 81; Olympus America, Melville, NY) equipped with an analog video camera (Olympus DP30BW) and a LC-PolScope retardance imaging system (Cambridge Research and Instrumentation, Woburn, MA) that combines liquid crystal-based polarizing light technology and data acquisition/analysis software to display birefringence, an inherent optical property of highly ordered molecules such as spindle microtubules. This noninvasive technology previously has been used to indicate the presence of birefringent spindles in human oocytes (5, 8, 24–27).

## ICC

After being incubated at 37°C, RT, or 4°C, MI, TI, and MII oocytes were attached to polylysine-coated coverslips and fixed in 2% (w/v) paraformaldehyde with 0.05% (v/v) Triton X-100 in phosphate-buffered saline (pH 7.3) for 30 minutes. The oocytes were blocked overnight with 2% (w/v) bovine serum albumin, 0.1 M glycine, and 5% (w/v) dry milk in phosphate-buffered saline at 4°C, and then incubated with  $\beta$ -tubulin antibody (1:200; Sigma, St. Louis, MO) for 1 hour at 37°C in a humidified chamber. Antibody binding subsequently was visualized following incubation of oocytes with an anti-mouse Alexa Fluor 488 antibody (1:750; Molecular Probes, Eugene, OR). Chromatin was counterstained with mounting solution containing 10 mg/mL Hoechst 33342 (blue) or propidium iodide (PI; Sigma; red). Fluorescence was evaluated using a Leica DMR fluorescent microscope (Manufac, Wetzlar, Germany) at a magnification of  $\times$ 400–1000. Images were captured and processed using a SPOT RT Slider digital camera and software (Diagnostic Instruments, Sterling Heights, MI).

## Vitrification/Warming Protocol

For vitrification, another batch of denuded MII oocytes that were collected and processed as described earlier were

incubated with 20  $\mu\text{L}$  M-199 HEPES-buffered medium (M-199-H)/12% (SSS) (w/v) for 1 minute before the addition of 20  $\mu\text{L}$  of equilibration solution (7.5% [v/v] ethylene glycol, 7.5% [v/v] dimethylsulfoxide [DMSO], 12% [w/v] SSS in M-199-H). After 2 minutes, an additional 20  $\mu\text{L}$  of equilibration solution was added to each oocyte sample. After 2 minutes, oocytes were transferred to 20  $\mu\text{L}$  of fresh equilibration solution for 3 minutes. The oocytes subsequently were divided into 20- $\mu\text{L}$  drops of vitrification solution (15% [v/v] ethylene glycol, 15% [v/v] DMSO, 0.5 M sucrose, 12% [w/v] SSS in M-199-H) to be incubated at 22°C for 5, 10, and 90 seconds. During the final 90 seconds in vitrification solution, oocytes to be cryopreserved were loaded onto a CryoTop (Kitazato, Japan) and submerged in liquid nitrogen. CryoTops were maintained in liquid nitrogen until oocyte warming.

For warming, CryoTops were removed from liquid nitrogen and tips containing oocytes were submerged rapidly in warming solution (1.0 M sucrose, 12% [w/v] SSS in M-199-H). Oocytes were then transferred into 20- $\mu\text{L}$  aliquots of warming solution for 2 minutes, followed by two 2-minute incubations in 20  $\mu\text{L}$  of dilution solution (0.5 M sucrose, 12% [w/v] SSS in M-199-H), and then three 2-minute rinses

in 20  $\mu\text{L}$  of wash solution (12% [w/v] SSS in M-199-H). All solutions were maintained at 22°C.

### Statistical Analysis

Nonparametric outcome measures were compared using chi-square analysis. A *P* value < .05 was considered a statistically significant difference.

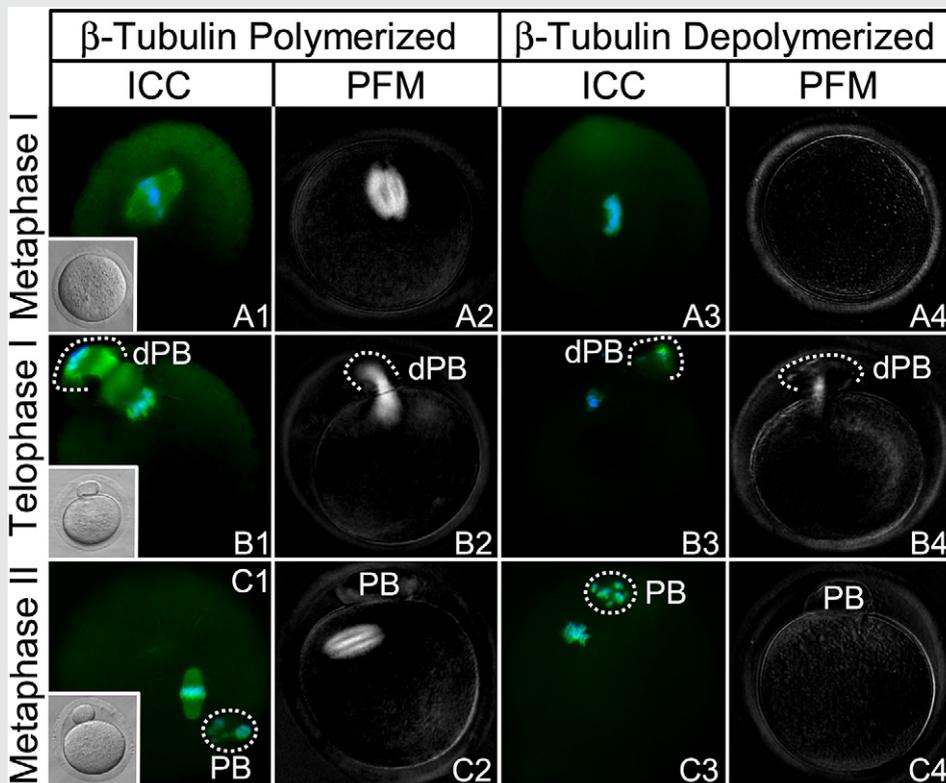
## RESULTS

A total of 514 mouse oocytes were used in this study, including 164 at TI, 168 at MI, and 182 at MII. At 0 hours and 37°C, all oocytes contained spindles that could be visualized by PFM, and ICC confirmed expression of  $\beta$ -tubulin in the spindles of oocytes at all three meiotic stages (Fig. 1). After 10 minutes at RT, 80%–100% of oocytes at each meiotic stage had spindles present as detected by both PFM and ICC (Figs. 2–4).

### MI Oocytes

As shown in Figure 2, after 10 minutes at RT, 100% of the meiotic spindles in MI oocytes were detected by both PFM and ICC. After 10 minutes at 4°C, though, the percentage of

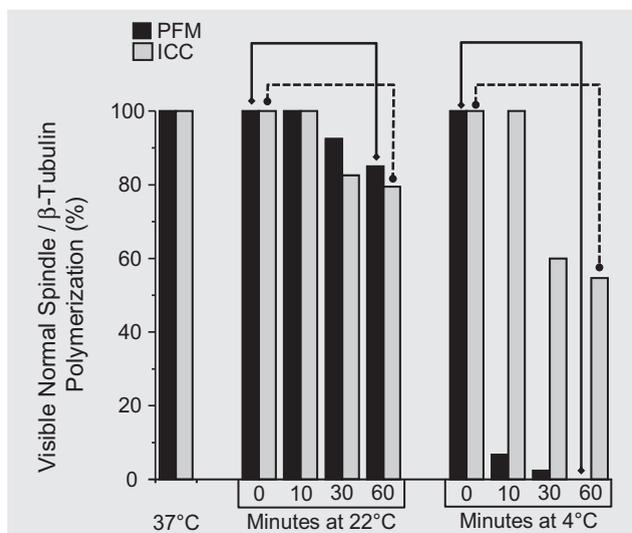
**FIGURE 1**



The presence and absence of tubulin polymerization in mouse oocytes detected using ICC and PFM. Using ICC, polymerized and depolymerized meiotic spindles were detected in MI oocytes (A1 and A3, respectively), TI oocytes (B1 and B3, respectively), and MII oocytes (C1 and C3, respectively). Using PFM, black-and-white images were obtained of polymerized and depolymerized meiotic spindles in MI oocytes (A2 and A4, respectively), TI oocytes (B2 and B4, respectively), and MII oocytes (C2 and C4, respectively). dPB = divided polar body; PB = polar body. All images are at a magnification of  $\times 400$ , except for the inset boxes shown for the A1, B1, and C1 panels that represent images of MI, TI, and MII, respectively, under light microscope. The dotted outlines indicate the polar body membrane.

Gomes. Temperature and oocyte meiotic stages. *Fertil Steril* 2012.

FIGURE 2



Percentages of meiotic spindles visualized in metaphase I oocytes analyzed by PFM and ICC. Oocytes were incubated at 37°C, RT, or 4°C for 0, 10, 30, and 60 minutes before being assayed.

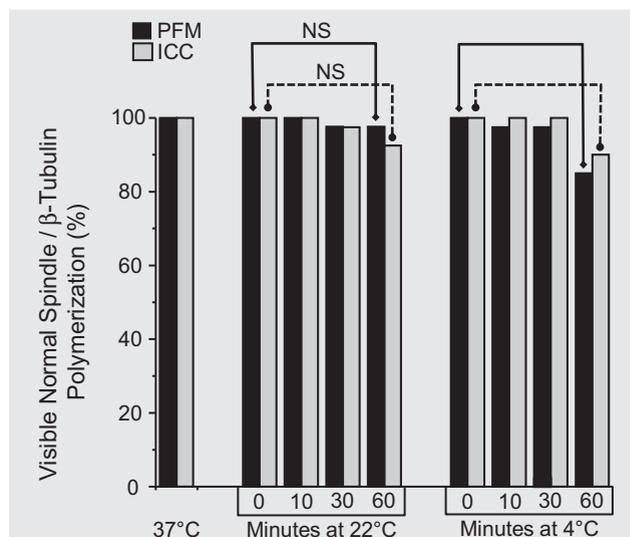
Gomes. Temperature and oocyte meiotic stages. *Fertil Steril* 2012.

spindles detected in MI oocytes by PFM (6.7%) was markedly lower than the percentage of  $\beta$ -tubulin positive MI spindles revealed by ICC (100%,  $P < .0001$ ). The percentage of spindles detected and the number of  $\beta$ -tubulin-positive spindles detected decreased with time at RT (22°C) or 4°C, but the decrease was markedly faster at 4°C than at 22°C. For example, at 4°C, the percentage of spindles detected by PFM decreased from 100% at 0 minutes to 0 by 60 minutes ( $P < .0001$ ), while the percentage of  $\beta$ -tubulin-positive MI spindles revealed by ICC decreased from 100% at 0 minutes to 54.7% at 60 minutes ( $P < .001$ ). On the other hand, the percentage of meiotic spindles detected by PFM after 60 minutes at RT decreased to 79.5% ( $P < .01$ ).

### TI Oocytes

Similar to our observations with MI oocytes, after 10 minutes at RT, 100% of the meiotic spindles in TI oocytes could be detected by PFM and ICC (Fig. 3). However, TI oocyte spindles were able to tolerate cold far better than MI oocytes. The percentage of spindles detected in TI oocytes at 4°C after 10 minutes using PFM and ICC was comparable (97.5% vs. 100%, respectively). Although the effect of cold on TI oocytes was less dramatic than on MI oocytes, the reductions became significant by 60 minutes (PFM at 0 minutes = 100%, PFM at 60 minutes = 85%,  $P < .01$ ; ICC at 0 minutes = 97.5%, ICC at 60 minutes = 90%,  $P < .01$ ; Fig. 3). Spindle visualization by PFM and ICC did not decrease significantly at RT ( $P < .05$ ) (Fig. 3). For TI oocytes ( $n = 146$ ) that underwent vitrification and warming,  $\beta$ -tubulin expression was detected in 93% of spindles analyzed at the start of warming, and in 100% of spindles after 60 minutes of warming (data not shown;  $P < .05$ ).

FIGURE 3



Percentages of meiotic spindles visualized in telophase I oocytes analyzed by PFM and ICC. Oocytes were incubated at 37°C, RT, or 4°C for 0, 10, 30, and 60 minutes before being assayed. NS = not significant.

Gomes. Temperature and oocyte meiotic stages. *Fertil Steril* 2012.

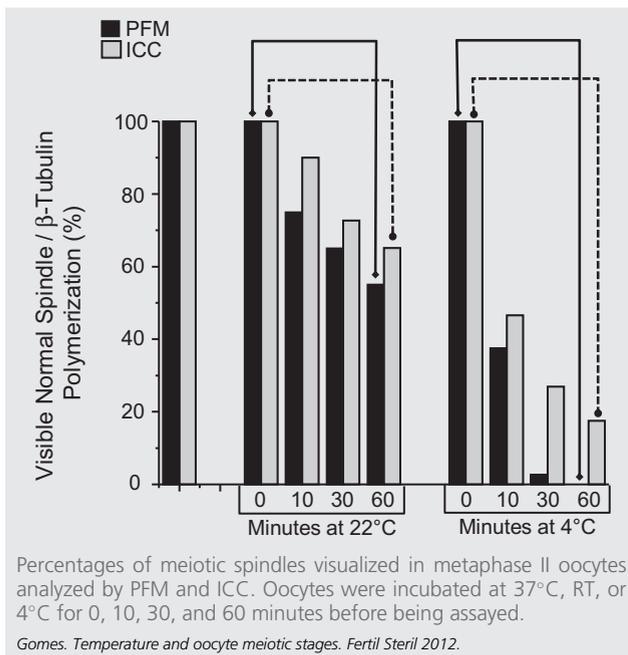
### MII Oocytes

MII oocyte spindles were less stable at RT than MI and TI oocytes. Indeed, after 60 minutes at RT, the percentage of meiotic spindles in MII oocytes detected by PFM decreased to 55% ( $P < .001$  vs. 0 minutes; Fig. 4). However, MII oocyte spindles showed a dramatic time-dependent reduction at 4°C. As shown in Figure 4, after 10 minutes at 4°C, there was not a large difference between the percentages of spindles detected by PFM (37.5%) and the percentage of  $\beta$ -tubulin-positive spindles detected by ICC (46.6%), with both showing significant reductions. After 60 minutes at 4°C, no spindles could be observed with PFM ( $P < .0001$  vs. 0 minutes), whereas 17.5% of  $\beta$ -tubulin-positive spindles could still be detected by ICC ( $P < .0001$  vs. 0 minutes). Furthermore, vitrified MII oocytes ( $n = 122$ ) exhibited a significant time-dependent increase in expression of  $\beta$ -tubulin, with no spindles expressing  $\beta$ -tubulin at the start of warming and 90% of spindles expressing  $\beta$ -tubulin after 60 minutes of warming (data not shown;  $P < .0001$ ).

### DISCUSSION

In the present study, PFM and ICC were used to characterize differences in spindle depolymerization in oocytes of various stages of meiosis exposed to different temperatures. Overall, spindle depolymerization was detected in oocytes at all meiotic stages, except TI oocytes at RT, and depolymerization was found to be time-dependent. TI oocytes showed less spindle depolymerization than MI and MII oocytes during cooling to RT, 4°C, and vitrification. Chen and colleagues (20) detected a time-dependent repolymerization of microtubules after warming of mouse oocytes. However, to our knowledge, the present study is the first to specifically analyze meiotic

FIGURE 4



spindle changes in relation to temperature variations and oocyte meiotic stage. Importantly, the presently reported observations indicate that the stability of tubulin polymerization, and thus spindle organization, is not constant through meiotic stage transitions. Rather, there is especially low susceptibility to cold-induced depolymerization during the TI stage.

In the various stages of meiosis, post-translational modifications of tubulin, including acetylation, have the potential to influence the stability of the meiotic spindle. Moreover, the presence of acetylated microtubules during meiosis and mitosis has been associated with a cell-cycle-specific pattern (28). Such modifications could explain the differences in spindle stability observed in response to temperature at different meiotic stages. However, further studies are needed to better understand the molecular and structural details of TI tubulin polymerization in relation to depolymerization induced by reduced temperatures.

In an elegant study by Montag and coworkers (29), spindle dynamics of human oocytes were recently observed during meiotic progression at physiologic temperature. Video sequences showed that the transition from MI to MII (encompassing the TI stage) included the complete disappearance of the meiotic spindle for 40–60 minutes. It has not been confirmed whether this observation is representative of an intra-oocyte biological process or a limitation of live cell imaging when intra-oocyte structures (like spindles) are moving within the cytoplasm, potentially out of the imaging view. However, if this disappearance is part of the physiology of oocyte development, as it appeared to be, then the absence of the spindle apparatus in some oocytes may be an indicator that those oocytes may have progressed through a developmental stage of meiosis, rather than experiencing a cellular disturbance.

Because in vitro-matured oocytes were used in this study, it remains unclear whether the observations of spindle polymerization/depolymerization made are representative of what occurs in oocytes that mature in vivo. It has been shown previously that human oocytes matured in vitro have an increased number of meiotic spindle and chromosome disturbances compared to oocytes matured in vivo, even with exposure to low temperatures (5, 26, 27). Moreover, Huang and coworkers (30) demonstrated that survival rates and the incidence of spindle and chromosomal abnormalities were similar between mouse vitrified oocytes matured in vitro and in vivo. Although it is important to further identify similarities and differences between in vivo and in vitro human matured oocytes, it is also important to recognize the limitations associated with the acquisition and analysis of the materials associated with each paradigm.

In the present study, meiotic spindles were visualized using PFM and ICC. However, there are technical limitations associated with each of these methods. For example, although the fixation and staining of samples in preparation for ICC facilitates a detailed analysis of spindles and chromosomes, only a snapshot of a particular oocyte is obtained for each sample (2, 16, 18). In contrast, PFM uses transmitted polarized light to provide a dynamic analysis of spindles before and after exposure to an experimental perturbation. Moreover, because PFM does not require fixation and staining, it provides a noninvasive imaging method without threatening oocyte viability (18). However, PFM live-cell imaging does have a limitation in that it is possible for mobile spindle structures to move into regions within the oocyte being examined that are outside of the imaged area. To prevent missing mobile spindles, we manually acquired each PFM image by transversing focal planes through the entire oocyte. As a result, the data collected for our TI and MII oocytes using ICC and PFM were relatively concordant. Moreover, our previously collected real-time observations of MII oocytes using PFM are consistent with data obtained from oocytes that were fixed and analyzed using ICC in the present study (2). Similarly, Chen and colleagues (20) also reported morphologic features associated with the spindle apparatuses of mouse oocytes imaged by polarized light microscopy before vitrification and warming that were consistent with ICC data. However, human MII spindle evaluations obtained using PFM by Coticchio and coworkers (25) were not consistent with confocal analysis. In their work, retardance measurements were found to have limited predictive value regarding the degree of spindle fiber order. Similarly, results regarding MI oocytes obtained from PFM and ICC in the present study were found to be discordant. This discordance could be due to a premature order of tubulin in MI oocytes. Correspondingly, it has been reported previously that the meiotic spindle in early prometaphase I may be relatively unordered. This circumstance is associated with reduced bi-refringence, and thus, can be more difficult to document by PFM (20).

In summary, the present study demonstrated that there are significant differences in microtubule depolymerization patterns in response to cooling between MI, TI, and MII mouse oocytes matured in vitro, with the most striking difference being observed between TI and metaphase oocytes. Notably, meiotic

spindles in TI showed less depolymerization at RT and at 4°C than did spindles in MI and MII oocytes. This observation may be very significant clinically given that both TI and MII oocytes have polar bodies and can be very difficult to differentiate morphologically by light microscopy. Moreover, a difference in sensitivity for polymerized tubulin present in TI oocytes vs. MII oocytes also was observed following vitrification and warming. Thus, it would be of interest to determine whether these differences in spindle susceptibility and temperature-induced depolymerization affect oocyte cryopreservation and subsequent cryosurvival, fertilization, and embryo development, as well as the rate of healthy live births. Therefore, future studies using model systems and human oocytes will be necessary to determine the functional importance of the reduced susceptibility of TI oocyte tubulin to the low-temperature-induced depolymerization observed in this study.

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