Comparative analysis of the metaphase II spindle of human oocytes through polarized light and high-performance confocal microscopy

Giovanni Coticchio, Ph.D.,[a] *Raffaella Sciajno, B.Sc.,*[a] *Karla Hutt, Ph.D.,*[b] *John Bromfield, Ph.D.,*[c] *Andrea Borini, M.D.,*[a] *and David F. Albertini, Ph.D.*[c]

[a] Center for Reproductive Health, Tecnobios Procreazione, Bologna, Italy; [b] Department of Genetics Cell Biology and Development, University of Minnesota, Minneapolis, Minnesota; and [c] Department of Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, Kansas

Objective: To determine whether Polscope analysis can predict different spindle and chromosome configurations of the oocyte metaphase II (MII) spindle.
Design: Comparison of Polscope and confocal microscopy analysis of the MII spindle.
Setting: Private IVF unit.
Patient(s): Women undergoing IVF treatment for male or unexplained infertility.
Intervention(s): Fresh and frozen-thawed mature oocytes were analyzed through the Polscope and, immediately afterward, fixed for confocal microscopy assessment.
Main Outcome Measure(s): Comparison of retardance values, derived from Polscope analysis, between spindles with different microtubule and chromosome configurations, defined by confocal microscopy evaluation. Measurements of spindle longitudinal axis through the Polscope and confocal microscopy.
Result(s): The mean retardance values of different categories of spindle configuration were not statistically significant in almost all cases, allowing only the identification of spindles with highly disorganized microtubules and chromosomes in frozen-thawed oocytes. In spindles with bipolar organization, the Polscope produced measurements of the spindle main axis which were in all cases statistically smaller compared with confocal microscopy evaluation.
Conclusion(s): Retardance measurements have limited predictive value of the degree of spindle fiber order and chromosome position in routine clinical settings. Also, under the conditions tested, morphometric evaluation of the spindle through the Polscope is not consistent with confocal analysis. This suggests that the Polscope may still be a rather inefficient method for assessing the metaphase II spindle and, as a result, for noninvasive oocyte selection. (Fertil Steril® 2010;93:2056–64. ©2010 by American Society for Reproductive Medicine.)

Key Words: Oocytes, metaphase II spindle, microtubules, confocal microscopy, Polscope, cryopreservation

The developmental fate of the mature human oocyte depends crucially on the metaphase II (MII) spindle. The principal function of this microtubular structure resides in its ability to assist chromatid segregation concomitantly with the extrusion of the second polar body (PB2), thereby ensuring completion of the meiotic process. In fact, the MII spindle plays a broader and more important role in oocyte physiology. Its subcortical position dictates a highly asymmetric second meiotic division, thereby minimizing the loss of organelles and molecules needed for the earliest stage of development, which accompanies the extrusion of the PB2, in a fashion similar to the first meiotic division (1, 2). Also, the MII spindle appears to be involved in processes not necessarily related to chromosome segregation and PB2 extrusion, as suggested by the fact that it represents a preferential localization site of Src-family protein tyrosine kinases, proteins essential for late stages of fertilization (3). As a highly dynamic and sensitive structure which depends on the finely regulated process of tubulin polymerization and depolymerization, it is not surprising that the MII spindle may be affected by diverse factors. Spindle perturbances can have intrinsic origins, as indicated by the fact that the large majority of oocytes from older women (40–45 years) display microtubular abnormalities and chromosome displacement compared with oocytes of younger women (20–25 years), in whom those anomalies are only sporadically represented (4). Inadequate culture conditions may represent an extrinsic source of spindle damage. In a landmark study almost two decades ago, using epiflorescence microscopy, Pickering et al.

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Reprint requests: Dr. Giovanni Coticchio, Tecnobios Procreazione, Via Dante 15 40125 Bologna, Italy (FAX: +39 051 2867512; E-mail: coticchio@tecnobiosprocreazione.it).

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(5) observed that in human mature oocytes cooled at room temperature for 10–30 minutes, the meiotic spindle suffers major structural alterations, including reduction in size, microtubular disorganization or disappearance, and chromosome dispersal. Upon rewarming to physiologic temperature (37°C), some of these spindle anomalies may disappear, but others persist, predisposing oocytes to developmental failure. Certain manipulations involved in IVF procedures are also thought to be particularly detrimental to the MII spindle integrity. For instance, the low-temperature preservation techniques of slow cooling and vitrification require the use of cryoprotectants whose action may affect spindle and chromosome configuration (6) as a consequence of acute osmotic stress and/or interference with the dynamics of tubulin polymerization. Furthermore, in vitro maturation has been identified as a possible source of spindle anomalies (7).

Detection and analysis of the MII spindle, therefore, could reveal fundamental information on the developmental competence of the mature oocyte and thereby provide criteria of quality and selection for improving the safety and efficiency of IVF treatment. Classically, the meiotic spindle may be visualized through fluorescence microscopy, which offers reliable and detailed information on microtubular structures and associated chromosomes. However, such a technique requires methods of preparation (fixation and staining) which are incompatible with the preservation of cell viability, and therefore cannot be adopted for oocyte selection in an IVF context. In the last several years, advances in polarized light microscopy have offered the opportunity to visualize the meiotic spindle noninvasively and in a dynamic fashion. In brief, the highly orderly structure of spindle microtubules generate the phenomenon of birefringence, which consists of the decomposition of a single incident beam of polarized light into two orthogonal rays. This creates a difference in contrast between the spindle and the rest of the cell which may be detected by imaging methods (e.g., the Polscope) that digitally amplify birefringence signals and, after computational manipulations, make quantifiable the degree of microtubule orientation within living cells.

In recent years, numerous studies explored the possibility of predicting oocyte developmental ability by monitoring MII spindle presence, position, or birefringence intensity (referred to as retardance). The majority of these studies indicated that absence of the spindle compromises the ability of the oocyte to fertilize and undergo normal preimplantation development (8–16), a conclusion which is consistent with the above-described spindle properties. This has led to the adoption of the Polscope as a tool for noninvasive oocyte selection by many IVF laboratories. Retardance values, which depend on the differential delay of the two decomposed orthogonal rays and are directly proportional to the density/number of spindle fibers in paracrystalline and parallel lattices (17), have also been suggested for predicting spindle normalcy and, as a consequence, oocyte developmental ability (18, 19). However, large segments of highly organized microtubular structures are not an exclusive feature of normally formed spindles, but are found also in spindles with major morphologic (e.g., tripolar) abnormalities or those displaying various degrees of chromosome dispersal. This property of microtubule fibers indicates a need to establish the range of capabilities of the Polscope to generate visual and quantitative data consistent with the organization of the MII spindle and associated chromosomes. Efforts toward this end are lacking or have been largely insufficient.

Therefore, and given the increasing application of the Polscope in clinical and research IVF, we performed a study with the objective of determining the correlation between Polscope analysis and actual configuration of the MII spindle assessed objectively through high-performance confocal microscopy. In particular, we tested whether retardance values can predict different spindle and chromosome configurations. Moreover, we compared the performance of the Polscope to measure accurately the pole-to-pole distance, which is believed to be an important indicator of MII spindle function (20). Before performing spindle analysis, a fraction of oocytes were frozen-thawed to assess the relative performance of the Polscope under conditions which are believed to affect spindle organization and are becoming progressively more widespread in human IVF laboratories, without intending to study cryopreservation per se.

MATERIALS AND METHODS

Source of Oocytes

The study, approved by the local Institutional Review Board, was conducted by using supernumerary oocytes. This material was donated by couples undergoing assisted reproductive technologies treatment for either male or unexplained infertility, after signing of an informed consent form. Controlled ovarian hyperstimulation was induced with long protocols using GnRH agonist and rFSH, according to the standard clinical procedures routinely used by the participating IVF clinic (21, 22). Ten thousand IU hCG was administered 36 hours before oocyte collection.

Oocyte Preparation

Two to three hours after oocyte retrieval, cumulus and corona radiata cells were removed from oocytes by a short exposure to HEPES-buffered medium (Sage IVF, Trumbull, CT) containing 20 IU/mL hyaluronidase (Sage IVF) and gentle aspiration in and out of a hand-drawn pipette and mechanical separation using denuding pipettes with a 130–170 μm diameter (Denuding Flexi-Pet; Cook, Brisbane, Australia). The denuded oocytes were then assessed for their meiotic maturation status. Oocytes with an extruded first polar body (PB1; presumably at the MII stage) and devoid of any sort of dysmorphism were immediately selected for meiotic spindle detection, and those without the PB1 and not displaying a germinal vesicle were cultured for a few hours to permit PB1 extrusion. Fixation for immunostaining was performed immediately after Polscope analysis.

Polscope Analysis of the Meiotic Spindle

Oocytes were placed in 5-μL drops of HEPES-buffered medium (Sage IVF) covered with mineral oil (Sage IVF) on a glass-bottomed culture dish (Will-Co Dish; Intracel, Herts, U.K.; or Labotech, Gottingen, Germany). Meiotic spindle visualization was performed at $\times$20 magnification of an inverted microscope with liquid crystal (LC) Polscope optics and controller (SpindleView; CRI, Woburn, MA), combined with a computerized image analysis system (SpindleView software). During Polscope analysis, temperature was maintained at 37°C with a heated stage. Oocytes were positioned trying to align the main axis of the spindle perpendicularly to the light path. To this end, by using the holding pipette of a micromanipulator integrated with the inverted microscope, oocytes were initially rotated until the PB1 was aligned with the oocyte's equatorial plane. In most cases this allowed the clearest and brightest visualization of the MII spindle. In a few cases, by gently releasing and reapplying the negative pressure generated by the holding pipette on the zona pellucida, oocytes were further rotated to achieve a better spindle visualization. Polscope images and raw data were saved rather than being immediately processed, to minimize oocyte exposure to suboptimal conditions. At a later stage, the spindle longitudinal axis was measured by drawing a pole-to-pole line and using a graduate glass slide as a reference scale. Mean $\pm$ SD retardance was measured throughout the spindle longitudinal axis at 1.0 μm intervals, and resulting values, generated by computer-assisted analysis, were saved for subsequent elaboration. The points immediately adjacent to the two ends of the spindle axis whose retardance values approached the ones of the surrounding background were excluded from the retardance measurements.

Cryopreservation

Oocytes were cryopreserved using a slow cooling method. All cryopreservation solutions were prepared using Dulbecco phosphate-buffered solution (PBS) (Gibco, Life Technologies, Paisley, Scotland) and a plasma protein supplement (PPS) (10 mg/mL final concentration) (Baxter, Vienna, Austria).

The equilibration solution contained 1.5 mol/L PrOH + 20% PPS in PBS, and the loading solution consisted of 1.5 mol/L PrOH + 0.3 mol/L sucrose + 20% PPS in PBS, as described by Fabbri et al. (23). The thawing solutions contained a gradually decreasing concentration of PrOH and a constant 0.3 mol/L sucrose concentration. They were prepared as follows: 1) 1.0 mol/L PrOH + 0.3 mol/L sucrose + 20% PPS; 2) 0.5 mol/L PrOH + 0.3 mol/L sucrose + 20% PPS; and 3) 0.3 mol/L sucrose + 20% PPS.

Freezing Procedure

Three hours after oocyte retrieval, cumulus-free oocytes were incubated in the equilibration solution for 10 minutes at room temperature (RT) and then transfered to the loading solution for 5 minutes. The oocytes were finally loaded in plastic straws (Paillettes Crystal 133 mm; Cryo Bio System, L'Aigle, France) individually or in small groups (maximum of three oocytes per straw). The temperature was lowered through an automated Kryo 10 series III biological freezer (Planer Kryo 10-1.7; Planer, Sunbury on Thames, U.K.) from 20°C to -8°C at a rate of 2°C/min. Manual seeding was performed at -8°C, and this temperature was maintained for 10 minutes to allow uniform ice propagation. The temperature was then decreased to -30°C at a rate of 0.3°C/min and finally rapidly brought to -150°C at a rate of 50°C/min. The straws were then directly plunged into liquid nitrogen at -196°C and stored for later use.

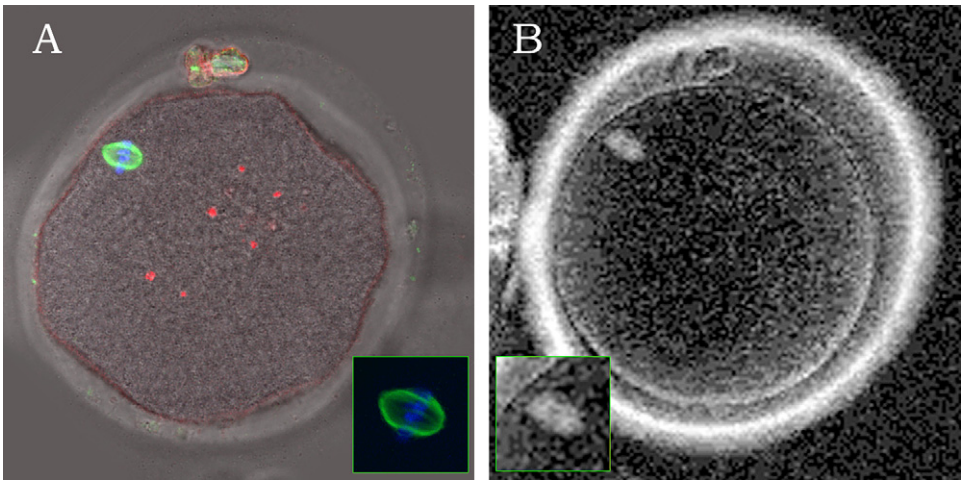
The thawing procedure was carried out at RT. Plastic straws were held in air at RT for 30 seconds and then transfered into a water bath at 30°C for 40 seconds. Stepwise cryoprotective agent dilution was performed by transfering the oocytes in thawing solution 1 for 5 minutes, then in solution 2 for an additional 5 minutes, and finally in solution 3 for 10 minutes before final dilution in PBS + 20% PPS for 20 minutes (10 minutes at RT and 10 minutes at 37°C). Before microscopy analysis, post-thaw culture was protracted up to 2 hours.

High-Performance Confocal Microscopy

Before Polscope examination and fixation for immunostaining, fresh oocytes were cultured for a total period of 4 hours in 20 μL drops of glucose-free medium normally used for embryo culture under warm mineral oil at 37°C in an atmosphere of 6% CO_2 in air, and frozen-thawed oocytes were incubated for up to 2 hours under the same culture conditions before spindle analysis. Oocytes were fixed and processed for microtubules and DNA immunofluorescence as previously described (24). Fixation and extraction was carried out for 30–60 minutes at 37°C in a microtubule-stabilizing buffer (100 mmol/L PIPES, 5 mmol/L $MgCl_2$, 2.5 mmol/L EGTA, 2% formaldehyde, 0.1% Triton-X-100, 1 mmol/L taxol, 10 U/mL of aprotinin, and 50% deuterium oxide [D_2O]) and stored until use in a blocking solution comprising PBS supplemented with 2% bovine serum albumin, 2% skim milk powder, 2% normal goat serum, 100 mmol/L glycine, 0.01% Triton-X-100, and 0.2% sodium azide. For immunostaining of microtubules, oocytes were first incubated with mouse monoclonal anti–$\alpha\beta$-tubulin (Sigma) diluted 1:100 in blocking solution for 1 hour at 37°C, followed by Alexa 488–labeled goat antimouse IgG (Invitrogen, Milan, Italy) diluted 1:800 in wash solution for 1 hour at 37°C. DNA was stained with Hoechst 33258 (1 μg/mL in blocking solution) for 30 minutes. Oocytes were mounted under cover slips without compression in medium containing 50% glycerol and 25 mg/mL sodium azide. Oocytes were analyzed using a Zeiss LSM Pascal confocal imaging system mounted on a Zeiss Axiovert II with UV (405 nm), HeNe (543 nm) and Argon (488 nm) laser excitation of fluorochromes. For every spindle, a complete Z-axis data set was collected at 0.7 μm intervals ($\sim$20 sections/spindle) using a $\times$63 oil immersion objective (numeric aperture 1.4). Spatial restoration and 3-dimensional projections for each Z-series data set were computed and analyzed using LSM 5 Image Browser.

FIGURE 1

Representative images of metaphase II spindle visualization through confocal microscopy (**A**) and Polscope (**B**).



Coticchio. Analysis of the oocyte metaphase II spindle. Fertil Steril 2010.

Statistics

Statistical comparison between fresh and frozen groups was carried out on pooled data using chi-squared analysis or *t* test for independent and paired data. A *P* value of $<.05$ was considered to be significant.

RESULTS

Assessment of Spindle Presence Through the Polscope

A total of 38 fresh and 78 frozen-thawed oocytes showing an extruded PB1 were used for a comparative analysis of the MII through polarized light and confocal microscopy. Fresh material included oocytes which showed the PB1 already at the time of cumulus removal (n = 22) or after a few hours of culture (n = 16). In agreement with the selection criteria of our clinical freezing program, all frozen-thawed oocytes were mature at the time of freezing and morphologically similar to the fresh material, i.e., showing no gross or even moderate anomalies, such as nonspherical shape, vacuoles, irregular zona pellucida, or pronounced granularity of the cytoplasm.

Only one fresh oocyte (2.6%) was devoid of a visible spindle after Polscope observation. After confocal observation, it appeared to have progressed, immediately after Polscope observation and before fixation, only to the telophase I stage and was not included for further analyses. In the frozen-thawed group, Polscope examination did not reveal a visible spindle in six oocytes (7.7%), whereas confocal microscopy showed that one of these oocytes possessed a disorganized spindle (category C; see below) and that the remaining oocytes displayed microtubule fibers that were highly disorganized but detectable (n = 2) or were absent (n = 3) (category D; see below).

Analysis of Spindle and Chromosome Configurations Through High-Performance Confocal Microscopy

After Polscope observation, fresh and frozen-thawed oocytes were immediately fixed and processed for confocal microscopy. This allowed a direct comparison of polarized light and confocal observation of the meiotic spindle (6) (Figs. 1 and 2). The chromosome segregation apparatus was found with different conformations of the microtubule and chromatin components, which were classified according to the following categories after high-performance confocal microscopy evaluation (Fig. 3) (6): A) bipolar spindle/aligned chromosomes: bipolar organization, with pointed or flattened poles, and microtubules converging at both poles and all chromosomes present and evenly aligned at the equatorial plate; B) bipolar spindle/nonaligned chromosomes: bipolar spindles, with pointed or flattened poles, and microtubules meeting at both poles, but chromosomes with varying degree of misalignment; C) disarranged spindle/aligned chromosomes: clusters of disorganized microtubules, multipolar spindles or spindles with microtubules not converging at one or both poles with chromosomes associated with microtubules and closely aligned; and D) severely disarranged or absent spindle/dispersed or absent chromosomes. These categories were represented in both fresh and frozen-thawed oocytes as illustrated in Figure 4. In fresh material, considering their overall number, oocytes matured in vitro contributed to confocal category A in a lower proportion (5 out of 16, 31.2%) compared with oocytes matured in vivo (8 out of 22, 36.4%).

Retardance of Different Spindle and Chromosome Configuration Categories

In fresh oocytes, the mean retardance derived from Polscope analysis did not differ statistically among spindles belonging to confocal categories A, B, C, or D ($P>.123$; Table 1). In particular, the mean retardance of categories A and B, whose spindles share a bipolar microtubule organization but differ in chromosome distribution (equatorial or dispersed, respectively) was 1.24 ± 0.24 and 1.30 ± 0.35, respectively. In

frozen thawed specimens, the mean retardance of confocal categories A, B, C, and D was 1.17 ± 0.27, 1.07 ± 0.31, 1.08 ± 0.47, and 0.83 ± 0.32, respectively (Table 1). A statistically significant difference was found only between groups A and D (P=.004). It is possible that the smaller sample size may have prevented detection of a significant difference between groups A and D in the fresh material as well. Comparing in vitro and in vivo matured oocytes, no significant difference was found in group A (1.22 ± 0.32 and 1.26 ± 0.20, respectively). In group B the difference (1.47 ± .032 and 1.18 ± 033) approached statistical significance (P=.068). The sizes of groups C and D did not allow a comparison between in vivo and in vitro oocytes.

Spindle Length Measurement Through Polarized Light and Confocal Microscopy

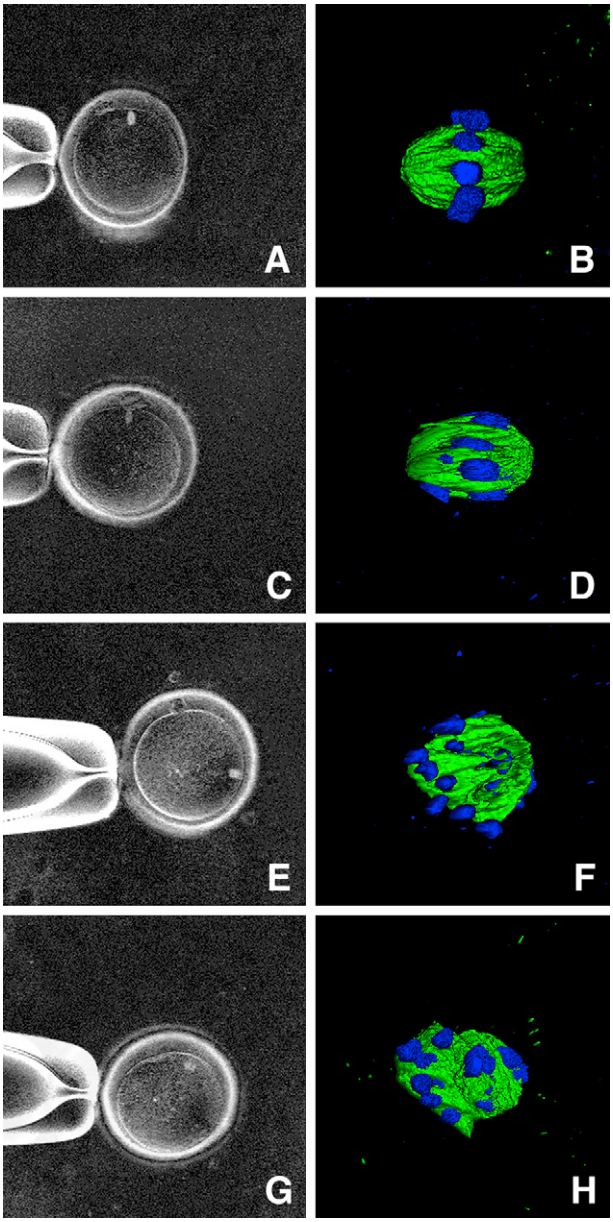
Spindles displaying a clear bipolar configuration after confocal analysis (categories A or B) were selected for assessing the relative ability of the Polscope to measure accurately the pole-to-pole length (Table 2). In fresh material, measurement of the spindle main axis through the Polscope produced mean lengths of 10.6 ± 2.1 μm and 10.7 ± 2.2 μm for categories A and B. This represented a statistically significant underestimation of the actual mean spindle size determined by confocal microscopy, which was 13.0 ± 1.9 μm and 13.5 ± 2.5 μm, respectively. A very similar trend was observed in frozen material, in which estimation of pole-to-pole distance through the Polscope generated values of 8.9 ± 1.6 μm (A) and 9.1 ± 2.5 μm (B), whereas the measures obtained by confocal microscopy were considerably higher (12.6 ± 1.8 μm and 14.8 ± 3.5 μm, respectively). By confocal analysis, spindle length was not significantly different between groups A and B in fresh oocytes, whereas in frozen material the difference between these groups reached statistical significance. In the fresh material, group A spindle length in in vitro and in vivo matured oocytes was 9.5 ± 2.0 μm and 11.3 ± 1.9 μm, respectively, after Polscope assessment and 12.6 ± 1.1 μm and 13.3 ± 2.3 μm, respectively, after confocal analysis. In group B, Polscope yielded spindle lengths in in vitro and in vivo matured oocytes, respectively, of 10.6 ± 0.7 μm and 10.8 ± 2.8 μm, and confocal evaluation 13.1 ± 2.0 μm and 13.8 ± 2.8 μm. All such differences were not statistically significant.

DISCUSSION

In human IVF, the strategy of noninvasively assessing and selecting oocytes of superior developmental potential is attracting increased interest, given growing demands to make human IVF safer and more efficient. Conservative oocyte selection is gaining further importance as a result of the introduction of new methodologies, including in vitro maturation and low-temperature storage, which have not been fully optimized and may, under inappropriate conditions, jeopardize oocyte quality. Noninvasive detection and assessment of the meiotic spindle using the Polscope has been proposed as a possible answer to the question of oocyte selection, on the basis of the crucial role the spindle plays in meiosis, fertilization, cell polarity, and cleavage. In the present analysis, by applying high-performance confocal microscopy, we tested whether different spindle conformations may be discriminated by retardance measurement to evaluate the actual diagnostic value of the Polscope. The two microscopy techniques were also compared in their relative ability to measure

FIGURE 2

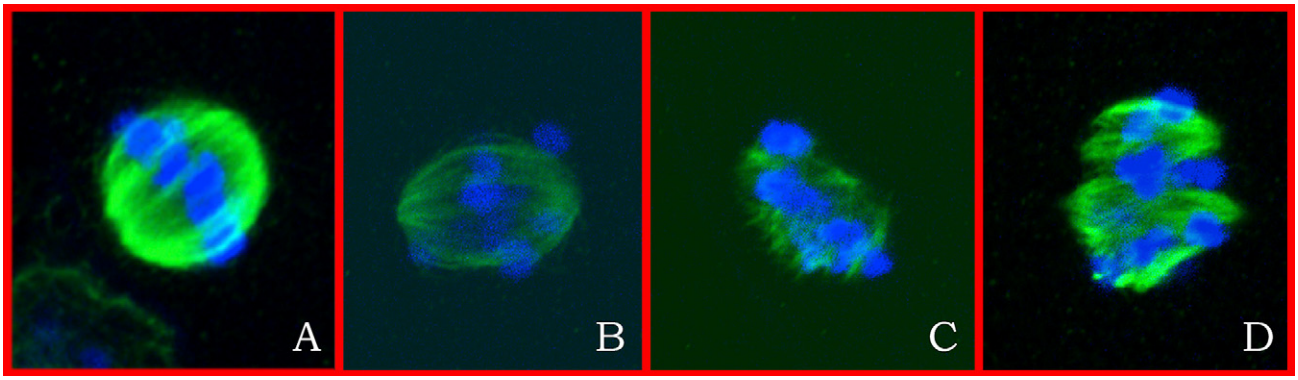
Polscope images (**A**, **C**, **E**, **G**) and confocal microscopy 3-dimensional reconstructions (**B**, **D**, **F**, **H**) of spindles presenting different microtubule and chromosome configurations. Oocytes were first observed through the Polscope and then immediately fixed for confocal analysis.



Coticchio. Analysis of the oocyte metaphase II spindle. Fertil Steril 2010.

FIGURE 3

Representative images of categories (**A**–**D**) of spindles showing different microtubule and chromosome configurations (see text for details). Microtubules were stained with secondary antibody conjugated with Alexa Fluor 488 (green), and chromosomes were visualized with Hoechst 33258 (blue).



Coticchio. Analysis of the oocyte metaphase II spindle. Fertil Steril 2010.

the spindle longitudinal axis, in consideration of the possible functional significance of this morphologic parameter.

As early as 1937 (25), polarized light microscopy was used to visualize the spindle during the mitotic or meiotic process in various cell types. More recently this technology has been adopted for the analysis of mammalian oocytes, after development of a commercially available version of this type of microscopy. In 2001, Wang et al. (9) published the first study performed on human oocytes with the Polscope, reporting a positive association between the presence of the MII spindle and fertilization ability after intracytoplasmic sperm injection.

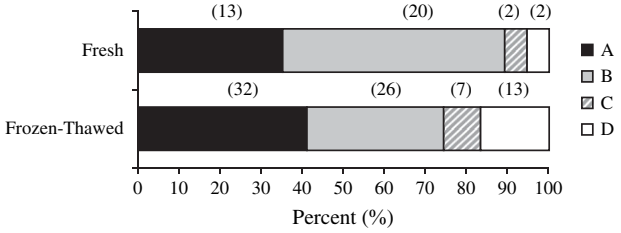
Concomitantly (8), the same group presented another study suggesting that oocytes displaying the MII spindle are more likely to develop to the morula and blastocyst stages. In the following few years, other reports (11, 12) essentially confirmed the initial observations of Wang et al. (8). These results have raised great interest from the IVF community, although it has also been realized that mere assessment of spindle presence appears to have a poor positive predictive value, because a large majority of fresh oocytes (generally >90%) with an extruded PB1 display a birefringent MII spindle. For such a reason, Polscope technology has been used in further investigations, which have suggested that spindle position relative to the PB1 may be indicative of the oocyte developmental potential (12, 26). The Polscope has found innovative application also because it allows repeated observation of the same specimen, again, in a completely noninvasive fashion. This has permitted the dynamic observation of the spindle in the course of in vitro treatments which may induce microtubule depolymerization and repolymerization, e.g., transient cooling to suboptimal temperature (18) or freezing-thawing (27, 28).

Together, despite a scarcity of clinical studies, these findings have encouraged the use of the Polscope as a method to detect the presence and location of the MII spindle and therefore to predict, to some extent, oocyte developmental ability. Technical limitations of polarized light microscopy do exist. Because this approach yields marginal image quality for human meiotic spindles, relevant properties of the MII spindle, such as chromosome positioning, cannot be detected, raising questions about the diagnostic value of Polscope analysis. High-performance confocal microscopy provides optimal resolution of MII spindle components but remains a destructive technique because it requires fixation.

Our present objective was to match Polscope results with those of confocal microscopy to better judge the relationship between microtubules and chromosomes that might reflect different developmental competences. Normally, spindle microtubules are arranged in a bipolar structure. Alternatively,

FIGURE 4

Relative representation of spindle categories **A**–**D** in fresh and frozen-thawed oocytes. Unlike fresh oocytes, the frozen-thawed group included six oocytes of category C (n = 1) and D (n = 5) in which Polscope examination had failed to identify a birefringent spindle. Oocyte numbers are in parentheses.



Coticchio. Analysis of the oocyte metaphase II spindle. Fertil Steril 2010.

TABLE 1

Comparison of mean ± SD retardance among spindles belonging to confocal categories A, B, C, and D. Fresh and cryopreserved oocytes were compared separately. Oocyte numbers are in parentheses.

	A	B	C	D
Fresh	1.24 ± 0.24 (13)	1.30 ± 0.35 (20)	1.46 ± 0.00 (2)	1.15 ± 0.17 (2)
Frozen-thawed	1.17 ± 0.27[a] (32)	1.07 ± 0.31 (25)	1.08 ± 0.47 (6)	0.83 ± 0.32[b] (8)

[a,b] $P=.004$.

Coticchio. Analysis of the oocyte metaphase II spindle. Fertil Steril 2010.

they display other, nonphysiologic, configurations yet retaining some degree of molecular order. Likewise, chromosomes may be positioned equatorially, partly misaligned, or widely dispersed (6). Whether these different configurations can be discriminated by the Polscope has been a matter of obvious interest, especially for clinical embryologists. Wang et al. (29) reported that 71% of in vitro matured oocytes displaying a birefringent spindle possessed a normal chromosome alignment after staining and analysis through confocal microscopy. They also found that in vitro matured oocytes not displaying a birefringent signal had abnormal spindles, misaligned chromosomes, or no sign of polymerized tubulin. This led the authors to conclude that simple visualization of a birefringent spindle provides a criterion for the selection of oocytes less prone to meiotic errors. We observed that only 35% of fresh oocytes showing a birefringent spindle in fact exhibited a bipolar microtubular arrangement and equatorial chromosome alignment (category A). Such a rate may have been determined also by the presence of in vitro matured oocytes, because, regarding their overall number, in vivo and in vitro matured oocytes were represented in different proportions in category A (36.4% and 31.2%, respectively). The proportion of frozen-thawed material oocytes with a birefringent spindle, and classified as A according to confocal analysis, was 44.4%. These rates are considerably smaller than the one described by Wang et al. (29) and argue against the notion that mere spindle birefringence is a good indicator of spindle normalcy and chromosome alignment. The incidence of bipolar spindles with regularly positioned chromosomes reported in the present study is small compared with our previous observations (30). This may be explained by the fact that most oocytes were derived from few patients and that the proportion of spindles with a normal configuration is influenced by the time of observation after thawing (Bromfield et al., unpublished data). However, experiments conducted in the mouse (20) are consistent with our conclusion that the Polscope is unable to define whether chromosomes are aligned equatorially (group A) or dispersed in various locations of the MII spindle (group B). This is in agreement with the fact that chromosomes are generally not detectable by polarization optics. Therefore, the Polscope presents utility for analyzing microtubule dynamics, but not for the analysis of chromosome behavior.

Novel (e.g., Oosight) or future (31) technical developments in polarized light microscopy will hopefully improve its utility to define more accurately meiotic spindle organization. It is also interesting to note that our data concerning frozen-thawed oocytes suggest that inability to detect a birefringent signal is not absolutely associated with an absence of polymerized tubulin. Furthermore, the fact that oocytes with spindles of category A, i.e., bipolar organization and aligned chromosomes, represent a minority is not surprising, because in vitro maturation (7), as in the case of part of the fresh material, or limited culture after freezing-thawing (Bromfield et al., unpublished data) have the potential to affect spindle organization.

TABLE 2

Comparison of Polscope and confocal microscopy measurements of the spindle longitudinal axis in categories A and B, i.e., showing a clear bipolar configuration. Confocal microscopy measurements between groups A and B were also compared separately in fresh and frozen thawed oocytes. Oocyte numbers are in parentheses.

	Fresh		Frozen-thawed	
	Polscope	Confocal	Polscope	Confocal
A	10.6 ± 2.1 (13)[a]	13.0 ± 1.9 (13)[b]	8.9 ± 1.6 (31)[e]	12.6 ± 1.8 (31)[f]
B	10.7 ± 2.2 (20)[c]	13.5 ± 2.5 (20)[d]	9.1 ± 2.5 (25)[g]	14.9 ± 3.5 (25)[h]

[a–h] $P<.001$.

Coticchio. Analysis of the oocyte metaphase II spindle. Fertil Steril 2010.

In the present investigation, we also tested the hypothesis that retardance values are predictive of MII spindle characteristics. Previously, Wang et al. (18) reported that maximum retardance of the spindle decreases as oocytes are cooled to suboptimal temperature (25°C). Retardance values are thought to reflect the density of microtubules and, therefore, the degree of order of the spindle, and cooling is known to cause depolymerization of microtubules (5). This has led to the assumption that retardance may be adopted as an indirect measure of spindle normalcy. Successive observations have attempted to establish a possible predictive significance of spindle retardance. For example, Shen et al. (15) found that high retardance values are positively correlated with a good pronuclear score as well as with transfer cycles giving rise to a pregnancy. In the present study, we found no correlation between different microtubule and chromosome configurations and retardance values. In particular, retardance appears to be unable to discriminate between spindles with a bipolar precisely organized configuration and aligned chromosomes (category A) from those having a highly disarranged microtubular structure (categories C and D in fresh oocytes and C in frozen oocyte). A statistically significant difference was found only between categories A and D in frozen-thawed material. This might be an effect of enhanced microtubular disarrangements, involving in any case a small percentage of oocytes, generated by the freezing-thawing process. In fresh material, it is possible that differences in the retardance of categories A and D may not have reached statistical significance because of the relatively smaller samples size. However, the possibility of identifying highly disorganized spindles (category D) by retardance measurements would aid in cases where the spindle is suspected to be particularly vulnerable to damage, as in older patients, in procedures involving cryopreservation, after in vitro maturation as shown here, or in toxicologic studies. In these cases, the discrimination of highly disorganized spindles could contribute to the design of improved culture or manipulation methods, as well as to the selection of gametes of higher developmental potential.

Finally, by comparison with confocal analysis, we assessed the ability of the Polscope to give a precise measurement of the pole-to-pole distance of the spindle. This evaluation was justified by the possibility that longitudinal spindle size represents a morphologic marker of oocytes quality, on the basis of the evidence that in experimental models the meiotic spindle appears shorter in oocytes prone to meiotic errors, owing to age (32) or xenobiotic treatment (20). We compared Polscope and confocal microscopy measurements in categories A and B, i.e., those including bipolar spindles, of both fresh and frozen-thawed oocytes, confirming in all cases that the Polscope underestimates the actual pole-to-pole distance by 2.5–5.8 μm. Such a discrepancy might depend on particular properties of spindle poles not amenable to polarized light detection, as suggested by an earlier study (33). Alternatively, this could reflect the difficulty during Polscope imaging of accurately positioning the spindle main axis orthogonally to incident light and thus to achieve an orientation-independent objective measurement. Other possible explanations may lie either in the lower resolution power of the $\times$20 objective used for Polscope analysis, which may introduce wider margins of measurements errors, or in possible modifications of the original spindle length caused by the fixation procedure that is required for confocal examination. Our fixation protocol involved the use of taxol and D_2O, agents which are known to stabilize microtubular structures and could therefore induce microtubule polymerization. Because exposure to these conditions is simultaneous with detergent-facilitated formaldehyde fixation, the immediate access of a protein cross-linker during detergent-effected dilution of soluble protein is unlikely to have modified spindle structure as a result of this type of specimen preparation. Moreover, MII spindles in in vitro matured murine oocytes have been reported to be identical in two independent studies involving fixation protocols with or without taxol (20, 34). In one of those studies, using mouse oocytes matured in vitro, Shen et al. (20) observed that spindle size evaluation is similar between immunofluorescence analysis and measurement performed with the Polscope. We suspect that this discrepancy between the present data and those of Shen et al. (20) is due to differences between mouse and human oocytes. Therefore, the reliability of spindle size measurement performed through the Polscope might also depend on inter- and intraspecies variance in spindle structures (34). Further considerations are suggested by the fact that, in the present study, spindle length values of fresh categories A and B, assessed with the Polscope, were approximately 2 μm smaller compared with data previously reported by Shen et al. (15). Such a difference might reflect methodologic diversities in the two analyses. Nevertheless, other factors might explain the discrepancy. In particular, it should be noted that our material was unselected and therefore presumably included several developmentally incompetent oocytes, whereas in the study of Shen et al. (15) spindle measurements were restricted to oocytes capable of fertilizing and cleaving. In this context, it is interesting to note that in the study of Shen et al. (15), oocytes with spindles of reduced size gave rise to zygotes with abnormal pronuclear pattern. In addition, another explanation may lie in the fact that the patients of the study of Shen et al. (15) were relatively younger (31–32 years) compared with ours (data not shown), a difference which could have led to a reduced spindle length in our study. In fact, observations in the mouse suggest that pole-to-pole distance of the MII spindle is shorter in older females (32). Furthermore, our confocal data revealed that in frozen-thawed, but not fresh, oocytes, spindle length was significantly smaller in category A (chromosomes aligned equatorially) compared with category B (chromosomes dispersed). Such a difference is difficult to interpret but could reflect highly complex dynamics of spindle depolymerization and repolymerization which occur during the first few hours of post-thaw culture (Bromfield et al., unpublished data).

In conclusion, our observations are not consistent with the assumption that qualitative detection of spindle birefringence is indicative of normal spindle organization and chromosome

distribution. We also tested the value of quantitative Polscope analysis, deducting that retardance measurements are informative on the degree of order or polymerization of microtubules only in cases of extreme structural disarrangement after cryopreservation. Finally, under the conditions tested, morphometric evaluation of the spindle through the Polscope is not consistent with confocal analysis. Collectively, this evidence suggests that the Polscope provides limited information, particularly in cases where whole patients' cohorts of oocytes are affected by major spindle anomalies or handling methods are suboptimal, leading to disruption of the microtubular organization (19). The Polscope, however, remains a valid approach to study spindle dynamics, owing to its absolute noninvasiveness. Currently, we are investigating the possibility that more advanced polarized light microscopy devices (e.g., the Oosight) may provide a better representation of the spindle constitution.

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