Zona pellucida birefringence in in vivo and in vitro matured oocytes

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Objective: To evaluate zona pellucida birefringence (ZPB) in immature and mature oocytes collected after controlled ovarian stimulation and to assess the influence of ZPB on oocyte development.
Design: Prospective study.
Setting(s): Private assisted reproduction centre.
Patient(s): Thirty patients undergoing intracytoplasmic sperm injection.
Intervention(s): The ZPB of mature and immature oocytes was evaluated using a polarization imaging software module, and the oocytes were classified as high birefringence (HB) or low birefringence.
Main Outcome Measure(s): The ZPB of in vivo and in vitro matured oocytes and its influence on spontaneous nuclear maturation in vitro, fertilization, and embryo quality.
Result(s): The percentage of HB oocytes was higher in immature than in mature oocytes (40.1 vs. 23.6%). Among immature oocytes, an increased percentage of HB in prophase-I stage oocytes compared to metaphase I stage oocytes was also observed (50.7 vs. 25.0%). However, the percentage of HB oocytes did not change when comparing oocytes before and after in vitro maturation for both prophase I and metaphase I oocytes. No influence of ZPB was observed on the spontaneous in vitro maturation potential. Exclusively for metaphase II retrieved oocytes, a positive influence of ZPB on fertilization (odds ratio [OR], 1.78; 95% confidence interval [CI], 1.27–2.49) and embryo quality (OR, 2.28; 95% CI, 1.04–4.99) was noted.
Conclusion(s): ZPB may be a useful tool to predict embryo quality for metaphase-II oocytes. Moreover, the completion of nuclear changes in the production of metaphase-II oocytes in vitro may not reflect their molecular maturity. (Fertil Steril® 2010;94:2050–3. ©2010 by American Society for Reproductive Medicine.)

Key Words: In vitro maturation, controlled ovarian stimulation, intracytoplasmic sperm injection, pregnancy, implantation, zona pellucida, birefringence

During controlled ovarian stimulation (COS), women are usually treated with an agonist or antagonist of GnRH to block the action of the pituitary, and their ovaries are stimulated with gonadotropins to induce the development and final maturation of multiple follicles (1). After stimulation and owing to a lack of synchronicity in maturation, oocytes at different stages of development are retrieved (2, 3). Of these, some immature oocytes (retrieved at either prophase I [PI] or metaphase I [MI] stages) have the potential for nuclear maturation and further development (4). Thus, rescue of these oocytes by spontaneous maturation in vitro may be one way to increase the number of embryos obtained from COS cycles, especially in cases of poor responders or in patients with an unsynchronized cohort of follicles (5).

Oocyte maturation remains a poorly understood process. It is generally defined as the time from the initiation of germinal vesicle breakdown to the completion of the nuclear changes leading to the expulsion of the first polar body (6). However, completion of the nuclear changes required to produce a metaphase II (MII) oocyte does not necessarily correlate with developmental competence and does not reflect the oocyte's molecular and structural maturity (2). Attempts have been made to promote maturation of immature human oocytes that have been retrieved from stimulated cycles. However, although successful fertilization, embryo development, and pregnancy have been reported (7–9), the literature on the developmental potential of these oocytes from COS cycles is still scarce.

It has been proposed that polarized light microscopy might enable the evaluation of subcellular oocyte features such as zona pellucida (ZP) birefringence (10, 11). The ZP is a unique extracellular coat that surrounds the maturing oocyte during ovulation, fertilization, and early embryo development (12). The ZP is a dynamic matrix composed of filaments organized in layers in differing orientation, and it has been proposed that properties of the zona layers might reflect the history of oocyte cytoplasmic maturation (8). A correlation between zona birefringence and embryo developmental potential has been previously demonstrated (13, 14).

Our goal in this work is to evaluate ZP birefringence in immature and mature oocytes collected after COS and to assess the influence of ZP birefringence on the rescue of oocytes by spontaneous maturation, fertilization, and embryo development.

MATERIALS AND METHODS

Patients

This study included 346 oocytes collected from 30 patients undergoing oocyte retrieval for intracytoplasmic sperm injection (ICSI). Written

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informed consent was obtained, in which patients agreed to share the outcomes of their cycles for research purposes. The study was approved by the local institutional review board.

Oocytes were classified according to their ZP birefringence: high birefringence (HB) or low birefringence (LB); they were also classified according to nuclear maturation status: metaphase II (n = 229), MI stage (n = 48) or PI stage (n = 69). The influence of ZP birefringence on spontaneous nuclear maturation in vitro, fertilization, and embryo quality was evaluated.

Controlled Ovarian Stimulation

Controlled ovarian stimulation was achieved by long-term pituitary downregulation using a GnRH agonist (Lupron Kit; Abbott S.A. Societé Française des Laboratoires, Paris, France) followed by ovarian stimulation with recombinant FSH (Gonal-F; Serono, Geneva, Switzerland). Follicular dynamics were followed by transvaginal ultrasound examination, to follow the follicular growth, starting on day 4 of gonadotropin administration. When adequate follicular growth and serum E_2 levels were observed, recombinant hCG (Ovidrel; Serono, Geneva, Switzerland) was administered to trigger final follicular maturation. Oocytes were collected 35 hours after hCG administration by transvaginal ultrasound ovum pick-up.

Preparation of Oocytes

After retrieval, oocytes were incubated in culture medium (G-1-V1; Vitrolife, Kungsbacka, Sweden) covered with mineral oil (Ovoil; Vitrolife) at 37°C and 6% CO_2 for 5 hours. Cumulus cells were removed with a 30-second exposure to *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid–buffered medium containing 80 IU/mL hyaluronidase (Irvine Scientific, Santa Ana, CA), after which coronal cells were manually removed using a finely drawn glass Pasteur pipette (Humagen Fertility Diagnostics, Charlottesville, VA). The denuded oocytes were then assessed for nuclear status. Oocytes that were observed to have released the first polar body were considered mature and used for ICSI; immature oocytes were cultured in vitro.

Rescue Spontaneous Maturation

Immature oocytes were classified as PI- or MI-stage based on whether a germinal vesicle was visible. Oocytes with no visible germinal vesicle were considered to be MI-stage cells. Immature oocytes were incubated in culture medium (G-1-V1; Vitrolife) at 37°C and 6% CO_2. After 24 hours in culture, oocytes that had undergone nuclear maturation and reached the MII stage were injected with sperm.

Zona Pellucida Imaging and Intracytoplasmic Sperm Injection

For ICSI, oocytes were placed individually in 4-μL droplets of buffered medium (G-Mops-V1; Vitrolife). Sperm were placed in a central 4-μL droplet of polyvinylpyrrolidone solution (Irvine Scientific, Santa Ana, CA) in a 50 × 40-mm glass culture dish (WillCo-dish; Bellco Glass New Jersey) covered with warm mineral oil (Ovoil; Vitrolife).

Immediately before sperm injection, oocytes were placed under an inverted microscope (Eclipse TE 300; Nikon, Tokyo, Japan) with a heated stage at 37.0 ± 0.5°C (SD) and observed at ×400 magnification. Oocytes were screened using a polarization imaging software module (OCTAX PolarAIDE; Octax, Herborn, Germany) to evaluate ZP birefringence. Individual images combining bright field and birefringence views were recorded by the imaging software. Depending on the intensity and uniformity of the birefringent inner zona layer, ZP birefringence was evaluated though an automatic scoring module. The oocytes were classified as having high or low ZP birefringence. To this end, 100 oocytes were previously evaluated. All ZP birefringence scores were recorded and the median value was calculated. Based on the median value (5.5), the oocytes were classified as having high or low ZP birefringence.

Assessment of Fertilisation, Embryo Quality, and Embryo Transfer

Fertilization was assessed 18 hours after ICSI, and normal fertilization was declared when two clearly distinct pronuclei were present. Embryo quality was evaluated under an inverted microscope (Eclipse TE 300; Nikon). The following parameters were recorded: number of blastomeres, fragmentation percentage, variation in blastomere symmetry, presence of multinucleation, and defects in the ZP and cytoplasm.

Embryo transfer was performed on the third day of development. High-quality embryos were defined as those having all of the following characteristics: 8–10 cells, less than 20% fragmentation, symmetric blastomeres, absence of multinucleation, colorless cytoplasm with moderate granulation and no inclusions, absence of perivitelline space granularity, and absence of ZP dysmorphism. Embryos lacking any of the above characteristics were considered to be of low quality.

Statistical Analysis

Results expressed as percentages were compared by the chi square or Fisher exact test, where appropriate. Residue normality was tested for all variables. Transformations were performed when necessary. To study the influence of the ZP birefringence on spontaneous maturation, fertilization, and embryo development, logistic regression models were constructed and the results were expressed as odds ratios (ORs), 95% confidence intervals (CIs) and *P* values. Results were considered to be significant at the 5% critical level ($P < 0.05$). Data analysis was performed using Minitab (version 14, Minitab Inc., State College, Pennsylvania), a statistical analysis program.

RESULTS

Spontaneous In Vitro Maturation and Zona Pellucida Birefringence

The overall number of retrieved oocytes was 346, of which 229 (66.2%) were at the MII stage, 48 (13.9%) were at the MI stage, and 69 (19.9%) were at the PI stage. Thirty-six (75.0%) MI-stage and 26 (37.6%) PI-stage oocytes matured in vitro ($P < 0.001$).

The percentage of HB oocytes was higher in immature than in mature oocytes (92/229 [40.1%] vs. 17/117 [23.0%]; P=0.007). Among immature oocytes, we also observed an increased percentage of HB in PI-stage oocytes compared with MI-stage oocytes (24/48 [50.0%] vs. 17/69 [24.6%]; P=0.020). However, no differences were found in the percentage of HB oocytes when comparing oocytes before and after rescue by spontaneous maturation for either PI (24/48 [50.0%] vs. 12/26 [46.1%]; P=0.609; before and after rescue maturation, respectively) or MI oocytes (17/69 [24.6%] vs. 8/36 [22.2%]; P=0.627; before and after rescue maturation, respectively). Finally, no effect of ZP birefringence status was observed in the potential for rescue by spontaneous maturation (OR, 1.02; 95% CI, 0.87–1.18; P=0.844).

Fertilization and Embryo Quality

Intracytoplasmic sperm injection was performed for all oocytes matured in vivo and in vitro. For MII-retrieved oocytes, a positive influence of the ZP birefringence score on fertilization (OR, 1.78; 95% CI, 1.27–2.49; $P < 0.001$) was noted. The percentage of fertilized oocytes was significantly higher among the HB oocytes compared with the LB oocytes (Table 1).

In addition for MII oocytes, the ZP birefringence score was a determinant of the likelihood of high embryo quality (OR, 2.28; 95% CI, 1.04–4.99; P=0.036). This result was confirmed by further analysis that which showed that the percentage of high-quality embryos was significantly higher among HB oocytes (Table 1). However, for immature oocytes, ZP birefringence had no effect on fertilization

TABLE 1

Fertilization rate and percentage of high-quality embryos from embryos derived from PI-, MI-, and MII-stage oocytes according to the ZP birefringence.

	PI oocytes			MI oocytes			MII oocytes		
ZP birefringence	HB	LB	*P*	HB	LB	*P*	HB	LB	*P*
Fertilization rate (%)	33.3	28.5	0.79	62.5	60.7	0.927	81.4	64.5	0.020
High-quality embryos (%)	25.0	25.0	1.00	40.0	35.3	0.848	63.6	44.2	0.05

Braga. Zona pellucida evaluation in human eggs. Fertil Steril 2010.

(OR, 1.03; 95% CI, 0.86–1.20; P=0.633) or embryo quality (OR, 0.99; 95% CI, 0.88–1.18; P=0.736).

DISCUSSION

In stimulated cycles, pharmacologic doses of gonadotropins create a supraphysiologic hormonal environment that induces the growth of a cohort of follicles, which, under natural conditions, would become atretic and regress (15). De Vos et al. (16) showed that COS leads to the retrieval of oocytes at various stages of meiotic maturity and that some of these oocytes may complete maturation spontaneously in vitro (9).

The identification of predictive markers for oocyte development potential before fertilization is one of the most studied areas in assisted reproduction techniques. Until now, only a few predictive noninvasive markers for oocyte quality have been identified on the basis of morphologic criteria, which can be assessed using conventional microscopy (17). The introduction of polarization light microscopy has enabled the noninvasive visualization of subcellular structures in oocytes (18), such as the ZP. To our knowledge, this study is the first to evaluate the ZP birefringence status in immature and spontaneously matured oocytes and its relationship to in vitro maturation, fertilization, and embryo quality.

A possible relationship between ZP birefringence and embryo quality has been previously discussed. Eichenlaub-Ritter et al. (11) reported a higher ZP birefringence in oocytes contributing to conception cycles when compared with those of nonconception cycles. A recent study demonstrated higher implantation, pregnancy, and live birth rates when embryos derived from high birefringence zona oocytes were transferred (19). Embryo development was also reported to be superior in embryos derived from high zona birefringence oocytes (13, 14, 17, 19). These studies are in agreement with our findings, which demonstrate that the ZP birefringence score influences both fertilization rate and embryo quality for MII oocytes.

However, the same result was not observed when spontaneously matured oocytes were evaluated. It has been previously demonstrated that the implantation rate of oocytes -matured in vitro is significantly lower than that of oocytes matured in vivo (16). In addition, in our experiment, oocytes were retrieved from COS cycles. It would not be surprising to find suboptimal developmental capacity in oocytes that failed to mature in vivo, despite exposure to supraphysiologic levels of exogenous gonadotropins. Therefore, although it has been previously demonstrated that zona birefringence status may affect oocyte development, this effect would be difficult to observe in spontaneously matured oocytes.

In the present study, a higher incidence of HB among PI and MI oocytes was noted when compared to MII oocytes. Moreover, the percentage of HB oocytes was higher in PI than in MI oocytes. These findings suggest that zona birefringence decreases as oocyte *in vivo* nuclear maturation takes place. However, our data showed that during in vitro spontaneous maturation, zona birefringence remains unaffected.

Montag et al. (19) described that embryos derived from high zona birefringence oocytes shows a better embryo development on day 3, but not on day 2 compared with low zona birefringence oocytes. Whereas high-birefringence ZP appears to be an indicator of high oocyte quality, we observed that it decreased during oocyte in vivo maturation.

Pelletier et al. (10) observed that cleavage-stage embryos show thinner ZP than both immature and mature oocytes. However, no differences in zona thicknesses were observed between immature and mature oocytes. Moreover, according to the authors, ZP birefringence increases proportionally with the increase on the zona layers' thickness.

A still unresolved question of zona imaging is related to the biologic cause of the variation of birefringence. The multilaminar structure of the ZP revealed by polarization microscopy is directly linked to the paracrystalline network structure of the zona, which is formed during oogenesis (20).

Previous studies indicate that properties of the zona layers might reflect the history of oocyte cytoplasmic maturation (8), whereas different development stages and culture conditions may alter the ZP architecture.

Our results show that more than 30% of the oocytes retrieved after COS are immature, which is consistent with previous findings (2, 3). Moreover we found that oocytes derived from the PI stage present lower maturation and developmental competence compared with those derived from MI stage oocytes. Considering that nuclear maturation consists of the germinal vesicle breakdown, the resumption of meiosis, and the first polar body extrusion, we assume that the exposure to the in vitro environment during a more complex phase of development may have important consequences for the potential of human oocytes.

It is well recognized that complete oocyte maturation depends on nuclear maturity and on the quality and maturity of the ooplasm and the plasma membrane system (21, 22). The oocyte cytoplasm undergoes dramatic changes in its structure during maturation (23). Although nuclear maturation can be accomplished easily in vitro, a concomitant maturation of the cytoplasm does not seem to occur properly, as indicated by the absence of specific proteins in the cytoplasm of oocytes matured in vitro (24). According to Van Blerkom (25), a higher incidence of meiotic errors may be a consequence of defective cytoplasmic maturation or loss of synchrony between nuclear and cytoplasmic maturation.

The relationship among oocyte cytoplasmic maturation and ZP birefringence is still unclear. Wang and Keefe (26) evaluated another

subcellular structure (the meiotic spindle) with a polarized light microscopy in living oocytes matured in vitro. The oocytes were then fixed and imaged by confocal microscopy. The authors observed that all oocytes that did not have birefringent spindle by the polarized microscope and showed abnormal or no spindles after the fluorescent staining.

Together with our findings, these studies suggest that, when recovered from COS cycles, in vitro oocytes matured spontaneously in vitro may have completed nuclear maturation, but full developmental competence may not have been achieved. When recovered after COS, immature oocytes may have a delayed maturation process. The reduced developmental competence compared with that in mature MII oocytes may be related to the inability of the follicles to respond to hCG administration synchronously with other larger follicles.

In summary, our study suggests that ZP birefringence may be a useful tool to predict embryo development for MII oocytes. Moreover, we suggest that in vitro completion of the nuclear changes required to produce MII oocytes may not reflect the molecular maturity of these oocytes. Nevertheless, the relationship between the oocyte cytoplasmic maturation and ZP birefringence still needs to be elucidated to confirm our findings.

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