

Article

Zona pellucida birefringence score and meiotic spindle visualization in relation to embryo development and ICSI outcomes



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Abstract

The meiotic spindle and the zona pellucida exhibit molecular order when imaged with polarized optics. This study aimed to investigate possible factors contributing to the zona pellucida birefringence score and meiotic spindle visualization, and to evaluate whether these structures may predict intracytoplasmic sperm injection outcomes. Oocytes were divided into groups according to zona pellucida birefringence and meiotic spindle visualization. In addition, the cycles were split into three groups based on the zona birefringence of transferred embryos. A positive correlation was observed between zona birefringence and meiotic spindle visualization. In addition, when the meiotic spindle was observed, the fertilization rate among oocytes with high or low zona pellucida birefringence was similar. Implantation and pregnancy rates were significantly higher when embryos derived from high birefringence oocytes were exclusively transferred ($P = 0.041$ and $P = 0.004$ respectively). Furthermore, the miscarriage rate was higher when embryos derived from low birefringence oocytes were exclusively transferred. On the other hand, the total dose of FSH negatively affected meiotic spindle visualization. Results show that selection of embryos based on zona pellucida and meiotic spindle imaging can significantly improve implantation and pregnancy rates. Moreover, the dose of FSH used for ovarian stimulation may affect the organization of the oocyte meiotic spindle.

Keywords: assisted reproduction, ICSI outcomes, meiotic spindle, zona pellucida

Introduction

Since the first healthy child was conceived via intracytoplasmic sperm injection (ICSI) (Palermo *et al.*, 1992), this technique has become increasingly popular as a means of infertility therapy. The main challenge for the success of ICSI is to produce viable embryos having high implantation potentials.

Oocyte quality has been regarded as a variable that influences the implantation potential of derived embryos. To date, there have been many published reports on the impact of oocyte morphology towards embryo development (Xia, 1997; Ebner *et al.*, 2000; Suppinyopong *et al.*, 2000; Wilding *et al.*, 2007).

The most frequently observed oocyte morphological variations are cytoplasmic, including changes in colour, granularity and homogeneity, as well as cytoplasmic incorporations. Extracytoplasmic variations are deviations from normal perivitelline space, zona pellucida colour and oocyte shape (Van Blerkom, 1990). However, the predictive value of criteria used in these studies is still controversial.

A promising approach has been proposed that uses spindle imaging as a predictor of oocyte quality (Wang *et al.*, 2001; Cohen *et al.*, 2004). Development of a polarized light microscope that

evaluates the birefringence of living cells enabled the evaluation of oocyte spindles without damaging the cell, as spindles are highly birefringent (Oldenbourg, 1996; Liu and Baker, 2000). The meiotic spindle controls chromosomal movement through different stages of meiosis, and is involved in various functions that are essential for fertilization and early post-fertilization events. These include the responsibility for proper chromosome segregation and genomic stability after oocyte activation (Cohen *et al.*, 2004; Eichenlaub-Ritter *et al.*, 2004; Rienzi *et al.*, 2005).

In addition to the meiotic spindle, polarized light microscopy enables the evaluation of other subcellular oocyte structures, such as zona pellucida birefringence (Pelletier *et al.*, 2004; Shen *et al.*, 2005). The zona pellucida is a unique extracellular coat that surrounds the maturing oocyte during ovulation, fertilization, and early embryo development (Familiari *et al.*, 2008). A correlation between zona birefringence and the potential of an embryo to develop to the blastocyst stage has been previously demonstrated (Rama Raju *et al.*, 2007).

During ovarian stimulation, women are usually treated with a gonadotrophin-releasing hormone (GnRH) agonist or antagonist to block the action of the pituitary, and their ovaries are stimulated with gonadotrophins to induce the development and final maturation of multiple follicles (Eldar-Geva *et al.*, 2003).

It has been suggested that hormonal stimulation protocols may induce oocyte chromosomal abnormalities (Munné *et al.*, 1997). A high daily FSH dose was also found to be associated with oocyte chromosome mosaicism (Katz-Jaffe *et al.*, 2005). In addition, a recent study provided evidence that ovarian hyperstimulation leads to oocyte meiotic segregation errors (Haaf *et al.*, 2008)

This study aimed to investigate possible factors that contribute to zona pellucida birefringence and meiotic spindle visualization, as well as to evaluate whether these two structures may predict embryo development and ICSI outcomes.

Materials and methods

Experimental design

This study was performed in 130 couples who were undergoing ICSI cycles, for the first time, from January 2008 to May 2008. A written informed consent was obtained, in which patients agreed to share the outcomes of their own cycles for research purposes, and the study was approved by the local review board.

Oocytes were divided into groups according to their zona pellucida birefringence and meiotic spindle visualization. These groups were defined as: high birefringence and spindle visualized (HBSV), high birefringence and spindle not visualized (HBSNV), low birefringence and spindle visualized (LBSV) and low birefringence and spindle not visualized (LBSNV). Fertilization rate and embryo quality were compared between the oocyte groups.

In addition, the cycles were split into three groups based on the zona birefringence of transferred embryos. Groups were defined as: a high birefringence-transfer group ($n = 20$), where only embryos derived from the high birefringence group were transferred; a combined-transfer group ($n = 74$), where embryos

derived from high birefringence and low birefringence groups were transferred; and a low birefringence-transfer group ($n = 36$), where only embryos derived from the low birefringence group were transferred. Implantation, pregnancy and miscarriage rates were compared among the three embryo transfer groups.

Furthermore, the influence of the female patient age, the number of follicles on the day of human chorionic gonadotrophin (HCG) administration, the number of retrieved metaphase II (MII) oocytes and the total dose of FSH on both zona pellucida birefringence and meiotic spindle visualization was evaluated.

Ovarian stimulation

Ovarian stimulation was achieved by long pituitary down-regulation using a GnRH agonist (Lupron Kit; Abbott S.A Société Française des Laboratoires, Paris, France) or antagonist (Cetrotide; Serono, Geneva, Switzerland), followed by ovarian stimulation with recombinant FSH (Gonal-F; Serono). The follicular dynamic was followed with ultrasound starting on day 4 of gonadotrophin administration. When adequate follicular growth and serum oestradiol concentrations were observed, recombinant human chorionic gonadotrophin (rHCG, Ovidrel; Serono) was administered to trigger final follicular maturation. Oocytes were collected 34–36 h after HCG administration by transvaginal ultrasound guided oocyte retrieval.

Sperm samples

Ejaculated spermatozoa were obtained by masturbation after 3–5 days of ejaculatory abstinence. After liquefaction of semen at room temperature, sperm samples were prepared by discontinuous density-gradient centrifugation or swim-up. For discontinuous density-gradients, the bottom fraction was aspirated and washed twice at 300 *g* for 8 min. For swim up, semen samples were diluted 1:1 with a HEPES-buffered modified HTF medium (Irvine Scientific, Santa Ana, USA) and incubated at 37°C for 1 h, allowing spermatozoa to move from the seminal plasma to the overlaid culture medium.

Preparation of oocytes

After retrieval, oocytes were incubated in culture medium (G1; VitroLife, Kungsbacka, Sweden), which was covered with mineral oil (Ovoil; VitroLife), at 37°C and 6% CO₂ for 5 h. Cumulus cells were removed with 30 s exposure to a HEPES-buffered modified HTF medium containing 80 IU/ml hyaluronidase (Irvine Scientific). After this, coronal cells were manually removed using a finely drawn glass Pasteur pipette (Humagen Fertility Diagnostics, Charlottesville, Virginia, USA).

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.

Zona pellucida imaging, meiotic spindle imaging and ICSI

For ICSI, oocytes were placed individually in 4 μ l droplets of buffered medium (human tubal fluid w/HEPES; Irvine Scientific, Santa Ana, USA). Spermatozoa were placed in a

central 4 μ l droplet of polyvinylpyrrolidone solution (PVP; Irvine Scientific) in a 50 \times 40 mm glass culture dish (WillCo-dish; New Jersey, USA) 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 $37.0 \pm 0.5^\circ\text{C}$ and observed at $\times 400$ magnification. Oocytes were screened using a polarization imaging software module (OCTAX PolarAIDE; Herborn, Germany) to visualize the meiotic spindle and to evaluate the zona pellucida birefringence. Zona pellucida evaluation was based on an automatic scoring module and the oocytes were classified as having a high or low zona birefringence.

Assessment of fertilization, embryo quality and embryo transfer

Fertilization was assessed 18 h 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: (i) number of blastomeres; (ii) fragmentation percentage; (iii) variation in blastomere symmetry; (iv) presence of multinucleation; and (v) defects in the zona pellucida and cytoplasm.

Embryo transfer was performed on day 3 of development. High-quality (grade A) embryos were defined as those having all of the following characteristics: 8–10 cells, <10% fragmentation, symmetric blastomeres, absence of multinucleation, colourless cytoplasm with moderate granulation and no inclusions, absence of perivitelline space granularity and absence of zona pellucida dysmorphism. Embryos lacking any of the above characteristics were considered to be of low quality.

For each couple, one to four embryos were transferred, depending on embryo quality and age of the female patient. Embryos derived from MII oocytes were given priority in selection for transfer.

Statistical analysis

Results are expressed as the mean \pm SD for numeric variables and proportions (%) for categorical variables. Mean values were compared by Student's *t*-test and proportions were compared by the chi-squared or Fisher's exact test, where appropriate.

To study the influence of zona birefringence on meiotic spindle visualization and the influence of female patient age, follicle number, number of retrieved MII oocytes and total FSH dose on zona pellucida birefringence and meiotic spindle visualization, multivariate regression models were conducted. Results were expressed as the regression coefficient (RC), odds ratios (OR), 95% confidential interval (CI) and *P*-value. Results were considered to be significant at the 5% critical level ($P < 0.05$). Data analysis was carried out using the Minitab (version 14) statistical program.

Results

General characteristics

The embryo transfer groups were similar with respect to all general characteristics (**Table 1**).

Oocyte zona pellucida birefringence status and meiotic spindle visualization

The overall number of retrieved MII oocytes was 1200, of which 235 (19.6%) were HBSV, 79 (6.6%) were HBSNV, 536 (44.7%) were LBSV and 350 (29.2%) were LBSNV. The logistic regression analysis showed that among the oocytes in which the meiotic spindle was visualized prior to ICSI, the incidence of high zona birefringence was increased (OR = 1.6; CI 95% = 0.24–10.81; $P < 0.001$).

Fertilization and embryo quality

When the meiotic spindle was observed, the fertilization rate among oocytes with high or low zona pellucida birefringence was similar. When the meiotic spindle was not visualized, the fertilization rate was significantly lower ($P < 0.001$); however, it also did not differ among oocytes with high or low zona pellucida birefringence (**Table 2**).

No significant difference in the percentage of high-quality embryos was observed when oocytes from different groups were injected (**Table 2**).

Implantation, pregnancy and miscarriage in relation to embryo transfer groups

Implantation and pregnancy rates were significantly higher when embryos derived from HB oocytes were exclusively transferred ($P = 0.041$ and $P = 0.004$ respectively). In addition, the miscarriage rate was higher when embryos derived from LB oocytes were exclusively transferred ($P = 0.021$; **Table 3**).

Factors contributing to the zona pellucida birefringence score and meiotic spindle visualization

When pituitary blockage with GnRH agonist or antagonist was studied, no statistical difference in the percentage of oocytes with meiotic spindle visualization [64.3% (507/789) versus 63.3% (264/417)], for agonist and antagonist respectively) or oocytes with high zona pellucida birefringence [25.9% (203/783) versus 26.1% (109/417)], for agonist and antagonist respectively,) was observed.

However, the logistic regression showed that spindle visualization, but not zona birefringence, was negatively influenced by the total FSH dose used for ovarian stimulation (**Table 4**). The women's age, follicle number on the day of HCG administration and number of retrieved MII oocytes did not influence oocyte birefringence or meiotic spindle visualization (**Table 4**).

Table 1. General characteristics of the three different transfer groups.

Groups	HB transfer	Combined transfer	LB transfer
<i>n</i>	20	74	36
Female age (years)	35.8 ± 4.9	34.9 ± 5.5	35.1 ± 5.4
Male age (years)	41.3 ± 7.98	40.4 ± 7.73	39.8 ± 6.97
Total dose of FSH (IU)	2363 ± 612	2389 ± 597.0	2504 ± 738
Follicles aspirated (<i>n</i>)	20.5 ± 9.7	17.0 ± 8.8	19.3 ± 10.2
Oocytes retrieved (<i>n</i>)	12.6 ± 8.2	13.1 ± 8.3	11.1 ± 8.3
Embryos transferred (<i>n</i>)	2.3 ± 1.3	2.9 ± 1.1	2.5 ± 1.2

Values are means ± SD.

There were no statistically significant differences between the groups. HB: high birefringence, LB: low birefringence.

Table 2. Fertilization rate and high quality embryo rate when oocytes with high or low zona pellucida birefringence and meiotic spindle visualization were injected.

	HBSV (<i>n</i> = 235)	HBSNV (<i>n</i> = 79)	LBSV (<i>n</i> = 536)	LBSNV (<i>n</i> = 350)	P-value
Fertilization (%)	79.1 ^a (186/235)	53.2 ^b (42/79)	75.7 ^a (406/536)	63.1 ^b (221/350)	<0.001
High-quality embryos (%)	76.6 (180/235)	77.2 (61/79)	78.4 (420/536)	74.6 (261/350)	NS

Values within rows without a common superscript differ. HB = high birefringence; LB = low birefringence; NS = not statistically significant; SNV = spindle not visible; SV = spindle visible.

Table 3. Implantation, pregnancy and miscarriage rates in the three different transfer groups.

Groups	HB transfer	Combined transfer	LB transfer	P-value
<i>n</i>	20	74	36	
Implantation rate ^a (%)	32.2 ^a	23.2 ^{a,b}	12.6 ^b	0.041
Pregnancy rate (%)	60.0 (12/20) ^a	50.0 (37/74) ^{a,b}	25.0 (9/36) ^b	0.004
Miscarriage rate (%)	0 (0/0) ^a	18.9 (7/37) ^{a,b}	33.3 (3/9) ^b	0.021

Values within rows without a common superscript differ. HB = high birefringence; LB = low birefringence

^aTotal implantation rate is the average of the implantation rate of each cycle.

Table 4. Multivariate regression analysis of factors contributing to the zona pellucida birefringence score and meiotic spindle visualization, with variables including: total FSH dose, age, follicle number on the day of human chorionic gonadotrophin (HCG) administration and number of retrieved metaphase II (MII) oocytes.

Response variable	Predictor variable	RC	OR	CI	P
Zona pellucida birefringence	Total dose of FSH	0.0001	0.98	0.77–1.19	NS
	Women's age	0.0018	1.00	0.98–1.14	NS
	Follicles	0.0213	1.02	1.02–0.98	NS
	Retrieved MII oocytes	-0.1515	0.86	0.72–1.02	NS
Meiotic spindle visualization	Total dose of FSH	-0.0013	0.63	0.51–1.77	0.004
	Women's age	-0.0481	0.95	0.88–1.03	NS
	Follicles	0.0213	1.02	0.98–1.06	NS
	Retrieved MII oocytes	-0.0279	0.97	0.91–1.04	NS

CI = confidence interval; NS = not statistically significant; OR = odds ratios; RC = regression coefficient.

Discussion

The identification of predictive markers for oocyte developmental potential prior to fertilization is one of the most studied areas in assisted reproductive techniques. Up to now, a few predictive non-invasive markers for oocyte quality have been identified on the basis of morphological criteria, which can be assessed using conventional microscopy (Ebner *et al.*, 2003). The introduction of polarization light microscopy enabled the non-invasive visualization of subcellular structures in oocytes (Oldenbourg, 1996), such as the meiotic spindle and zona pellucida birefringence.

The present study evaluated the oocyte zona pellucida birefringence status and meiotic spindle visualization, as well as its correlation with fertilization, embryo development and clinical outcome.

First, a positive correlation between the zona birefringence and spindle observation was found. The meiotic spindle is a highly dynamic structure, and its formation is regulated by hormones. Hormone stimulation initially triggers the resumption of meiosis by activating the maturation promoting factor (MPF), which induces chromosome condensation, nuclear envelope breakdown, and formation of the spindle (Cooper and Hausman, 2007). On the other hand, the zona pellucida forms during oogenesis. However, properties of the zona layers might reflect the history of oocyte cytoplasmic maturation (Qi *et al.*, 2002). Factors affecting the oocyte maturation process would therefore affect the organization of these two molecular structures, which would explain the observed relationship.

A higher fertilization rate was also observed for oocyte groups in which the meiotic spindle was visualized independently of zona pellucida birefringence status. In the MII-stage oocyte, chromosomes are aligned in the centre of the meiotic spindle, which is involved in many functions that are essential for the sequence of events leading to meiosis completion and fertilization (Rienzi *et al.*, 2005). On the other hand, although a thick and solid zona may protect the oocyte from mechanical stress during the microinjection procedure, it was demonstrated that ICSI has been successful in cases of zona-free human oocytes (Ding *et al.*, 1999; Takahashi *et al.*, 1999; Stanger *et al.*, 2001). However, implantation appears to be more affected by the absence of a zona (Hsieh *et al.*, 2001).

In the present report, after splitting the transferred embryos according to zona birefringence status, an influence of zona birefringence on ICSI clinical outcome was observed. For the group in which only embryos derived from high zona birefringence oocytes were transferred, implantation and pregnancy rates were considerably higher and the miscarriage rate lower. This is in comparison with groups having at least one embryo derived from low zona birefringence oocytes.

The findings also showed that when the meiotic spindle was observed prior to ICSI, the incidence of high zona birefringence was increased. It was previously reported that when only embryos derived from oocytes with detectable spindles were used, higher pregnancy and implantation rates were achieved (Madaschi *et al.*, 2007).

A possible role of the zona birefringence on embryo implantation potential has been previously discussed. Shen *et al.* (2005) reported a higher zona retardance in oocytes contributing to conception cycles when compared with non-conception cycles. A recent study demonstrated higher implantation, pregnancy and live birth rates when embryos derived from high birefringence zona oocytes were transferred (Montag *et al.*, 2008). Embryo development was also reported to be superior in embryos derived from high zona birefringence oocytes (Rama Raju *et al.*, 2007; Montag *et al.*, 2008).

In the present study, the percentage of high-quality embryos was not influenced by the meiotic spindle visualization or zona pellucida birefringence. It could be argued that both spindle and zona pellucida organization may be related to important aspects for embryo implantation, other than the morphology itself. Therefore, besides the standard morphology oocyte/embryo classification, this new approach for embryo selection may be essential for the identification of the embryo with the best implantation potential.

The mechanisms underlying meiotic spindle and zona pellucida protein organization remain poorly understood. It has been shown that meiotic spindle microtubules are highly sensitive to chemical and physical changes that may occur during ovarian stimulation and in-vitro culture (Lopes *et al.*, 1998; Zenzes *et al.*, 2001). In addition, as suggested by Keefe *et al.* (2003), artificial conditions associated with IVF might change the biophysical properties of zona pellucida proteins.

When possible factors affecting the zona birefringence status and meiotic spindle observation were evaluated, it was demonstrated that neither age nor the ovarian response to gonadotrophin stimulation affected the zona score or spindle visualization. The ovarian stimulation protocol, however, appeared to have a role in these figures.

As the use of GnRH antagonists enables ovarian stimulation without disrupting early follicular phase dynamics, leading to a lower stimulation and more appropriate response (Haaf *et al.*, 2008), we evaluated the meiotic spindle and zona pellucida of oocytes retrieved from cycles in which the pituitary blockage was achieved with a GnRH antagonist or agonist. It was observed that the percentage of meiotic spindle visualization and high birefringence zona pellucida oocytes did not depend on the pituitary blockage protocol. However, the total FSH dose was negatively correlated with spindle visualization.

The meiotic spindle plays a key role during meiotic non-disjunction and completion of meiosis (Rienzi *et al.*, 2005). Inaccuracies in the chromosome segregation machinery of oocytes are often involved in aneuploidy (Baart *et al.*, 2007), and preliminary observations suggest that aneuploidy in embryos may also be affected by ovarian stimulation regimens (Munné *et al.*, 1997; Katz-Jaffe *et al.*, 2005). Together with the present findings, this evidence suggests that hormonal ovarian stimulation may affect the organization of the oocyte meiotic spindle.

In conclusion, the results show that the selection of embryos based on zona pellucida and meiotic spindle imaging can significantly improve implantation and pregnancy rates. Moreover, the dose of FSH used for ovarian stimulation may affect the organization of the oocyte meiotic spindle.

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