Polarized light microscopy-detectable structures of human oocytes and embryos are related to the likelihood of conception in IVF

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Abstract

Purpose To clarify if birefringent structures of human oocytes and embryos, measurable by polarized light microscopy, have any value in predicting the chance of pregnancy in human in vitro fertilization and may help to identify the most competent oocytes and embryos.

Methods The inner layer of the zona pellucida (IL-ZP) and the meiotic spindle (MS) were analyzed by polarized light microscopy in 258 oocytes and in the 209 embryos deriving from them. Data obtained from 102 ICSI cycles with conception were compared with those obtained in 156 cycles without conception. The retardance and area of the IL-ZP, as well as the retardance, length of the major axis, and area of the MS were measured. Furthermore, polarized light microscopy parameters were related to the embryo morphological score by multiple regression analysis.

Result(s) The mean area of the IL-ZP of both oocytes and embryos was significantly lower in conception than in non-conception cycles ($p=0.0001$ for oocytes and $p=0.002$ for embryos). The area of the IL-ZP in embryos was significantly, inversely related to the embryo morphological score ($p=0.011$). The area, the major axis length and the retardance of the MS, as well as the retardance of the IL-ZP in oocytes and embryos were comparable in conception and non-conception cycles.

Conclusion The area of the IL-ZP of the human oocytes may represent a marker of oocyte competence, as oocytes with a low IL-ZP area are more frequently obtained in conception cycles. When measured in embryos, a low IL-ZP area identifies embryos with a high chance of implantation.

Keywords Polarized light microscopy · Zona pellucida · Meiotic spindle · Pregnancy · IVF

Introduction

The identification of reliable markers of oocyte competence represents an important issue in assisted reproductive technology, especially where the overproduction of embryos followed by their selection is not allowed or just where it is not considered a good strategy for the consequent need to cryopreserve many surplus embryos with practical and legal problems. The knowledge of oocyte-related variables having a good prognostic value in predicting the chance of the deriving embryo to achieve a pregnancy would allow the selective insemination of oocytes and would definitely limit the number of spare embryos.

The current level of knowledge about oocyte morphology includes only limited evidence: it has been shown that cytoplasm and zona pellucida appearance, as well as polar body morphology, may be helpful in assessing oocyte competence [1, 2], but their reliability in identifying the real oocyte developmental potential is still poor [3].

Polarized light microscopy (PLM) is a rather new non-invasive tool to analyze human oocytes; as no cell fixation is needed, no detrimental effects are induced on the female gamete by the procedure [4]. PLM allows the detection of anisotropic constituents of a cell (e.g. the microtubules of the meiotic spindle and the glycoproteins of the inner part of the zona pellucida), that once illuminated originate light beams deriving from light

Capsule The area of the zona pellucida inner layer is a marker of oocyte and embryo competence.

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polarization. These beams are differentially slowed down by well-organized, anisotropic cell structures, and the birefringent signals that are generated may be measured as “retardance” by a specific computerized image-analysis system [4].

Several studies on human oocytes reported that some birefringent characteristics of the meiotic spindle (MS) [5–8] and of the zona pellucida (ZP) [8–13] were associated with a high level of structural organization of the oocyte and could be positively related to the developmental potential of the embryo derived from it. These reports, however, were not fully convincing; especially those dealing with the MS were thoroughly criticized because the comparative analysis of the MS by confocal microscopy showed important discrepancies with PLM data [14].

In the present study we intended to verify if PLM could be helpful to understand oocyte competence and thus to select the oocytes having the best chance of originating high quality embryos and obtaining a pregnancy. Differently from previous reports, we extended PLM analysis also to the embryos, looking for dynamical changes in birefringent structures throughout the fertilization and cleavage processes, and studying if the birefringent structures of the embryos could be helpful in identifying its implantation potential.

Materials and methods

Patients

We studied 86 women undergoing an ICSI cycle for severe male factor ($n=68$, 79 %) or for a previous fertilization failure in IVF ($n=18$, 21 %). Some clinical characteristics of these women (age, BMI) and of their ICSI cycle (total administered FSH dose, number of retrieved oocytes, number of replaced embryos) were recorded. The ovarian sensitivity index (OSI = FSH dose/retrieved oocyte), a variable expressing the ovarian responsiveness to exogenous FSH, was also calculated [15].

The local ethical committee authorized the study and all enrolled patients gave their written, informed consent.

IVF procedure

Controlled ovarian hyperstimulation (COH) was carried out using recombinant FSH (Gonal-F, Merck-Serono, Switzerland) at individually tailored daily dose (100–450 IU) plus the GnRH-agonist Buserelin (800 mcg/day; Suprefact, Hoechst, Germany) according to a standard “long” protocol. Follicular growth was monitored by the serial measurement of circulating estradiol and by transvaginal US examination every second day from stimulation day 7. When at least two follicles reached 18 mm mean diameter, with appropriate estradiol levels, 10,000 IU human chorionic gonadotrophin

(hCG; Gonasi HP, IBSA, Switzerland) were administered subcutaneously. Ultrasound-guided oocyte retrieval (OPU) was performed 35–37 h later.

Aspirated follicular fluids were observed under stereomicroscope and cumulus-oocyte complexes (COCs) were washed in buffered medium (Flushing medium, Cook Ltd., Ireland). After incubation, the COCs were exposed to hyaluronidase (Synvitro Hyadase, Origio Medicult, Denmark) at a concentration of 80 IU/mL in HEPES buffered medium, and cumulus cells were removed by gentle mechanical aspiration with a denuding pipette in order to identify metaphase II (MII) oocytes. The retrieved oocytes were put in a specific medium (Fertilization medium, Cook Ltd., Ireland) and stored in controlled atmosphere (37 °C, 5 % CO₂) for at least 2 h.

At the same time, semen samples were collected by masturbation after 3–5 days of ejaculatory abstinence. Once complete liquefaction of the seminal plasma at room temperature was obtained, samples were prepared by discontinuous density gradient centrifugation and washed in buffered medium (Gamete medium, Cook Ltd., Ireland).

Immediately after cumulus cells removal, three MII eggs were selected to be inseminated; the selection criteria included the absence of cytoplasmic inclusions and/or zona pellucida abnormalities. Only three oocytes were inseminated in each ICSI cycle because this was imposed by the Italian law that regulated ART in the time period in which the study was performed (2009–first half of 2010). Oocytes were cultured individually in micro-drops of fertilization medium. ICSI was performed under polarized light guidance, placing the visible MS in the 6 or 12 o'clock position.

When normal fertilization occurred, zygotes were cultured individually in 10 µl of cleavage specific medium (Cleavage medium, Cook Ltd. Ireland). After 2 days, the embryo transfer (ET) of 1–3 embryos was performed using the Sydney catheter (Cook Ltd., Ireland). All the embryos obtained inseminating 3 oocytes were transferred in utero in agreement with the Italian law that regulated ART in the time period in which the study was performed. Luteal supplementation was accomplished using 90 mg/day vaginal progesterone (Crinone 8, Merck-Serono, Switzerland) for 15 days.

The diagnosis of clinical pregnancy was made when a positive urinary hCG test was obtained 15 days after ET and a transvaginal US examination (performed after two further weeks) confirmed the presence of at least one gestational sac in the uterus. Cycles in which couples obtained a clinical pregnancy were defined as conception cycles (CCs), whereas cycles with no pregnancy were defined as non-conception cycles (NCCs).

Polarized light microscopy (PLM)

During PLM examination, oocytes were placed on a glass-bottom dish (Willco Wells, The Netherlands) in a 10 µl drop

Fig. 1 PLM analysis of an oocyte. Manual measurement of MS major axis length

of buffered, pre-warmed medium covered by mineral oil (Culture Oil, Cook Ltd., Ireland) and kept on a 37 °C stage warmer under the polarization microscope (Oosight, CRI, USA). PLM images of the oocytes were collected at 400× magnification.

The day of ET, embryos were scored according to the previously published morphologic score by Holte et al. [16]; a few minutes before being transferred, embryos were placed on a glass bottom dish and analyzed by PLM at 400× magnification.

The Oosight Meta™ software coupled with automatic ZP and MS detector was used to acquire data for both oocytes and embryos. Briefly, oocytes were first observed

under bright field microscopy with focus plane adjustment, then filters for polarized light were applied and the software was calibrated for background acquisition. Once calibration was set, oocytes were analyzed at 400× magnification and gently rotated in order to visualize the MS and the ZP in the brightest focus plane. A similar procedure was followed to acquire data of embryos. The manual measurement of MS major axis length was performed drawing a line scan as previously described [6, 8] (Fig. 1). The image analysis was accomplished using the tools provided by the software: the “automatic-detection” button specifically identifies the boundaries of birefringent signals, thus determining the area of the birefringent structure, usually a barrel-shaped red area for the MS and a ring-like red structure for the IL-ZP (Fig. 2). All PLM images acquired on oocytes and embryos were recorded and later analyzed.

Although both MS and ZP obviously have a three-dimensional structure, the software provided direct bi-dimensional measurements of the area of these structures. The analysis of microtubular disposition, that provides informations that may be indirectly related to the three-dimensional structure of MS and ZP, was not applied in the present study.

Oocytes were subdivided according to the value of IL-ZP area into three subgroups: *thin-ZP oocytes* (IL-ZP area below the 33° percentile); *thick-ZP oocytes* (IL-ZP area above the 66° percentile); *medium-ZP oocytes* (intermediate-size IL-ZP area).

Fig. 2 PLM analysis of oocytes and embryos. Automated detection by Oosight™ software of birefringent structures of oocytes and embryos. (1) The bright signal corresponding to oocyte MS was automatically located and highlighted in red, obtaining data regarding retardance, length of the major axis and area; (2) the ring-like structure (in red)

corresponds to the automatic detection of the area and retardance of the inner layer of the zona pellucida (IL-ZP), that is particularly birefringent. Immediately before replacement into the uterine cavity, embryos underwent morphological scoring (3). Embryos with the highest score were selected for transfer and a PLM picture of IL-ZP was captured (4)

Table 1 Patients' clinical characteristics in conception (CCs) and non-conception (NCCs) cycles

	CCs	NCCs	<i>p</i>
N. of ICSI cycles	34	52	
Woman's age	34.4±0.8	35.3±0.8	ns
BMI	24.0±0.7	23.5±0.4	ns
N. of motile sperm after DGC-W (Mil/ml)	0.95±0.69	0.88±0.62	ns
Total FSH dose (IU)	2243±187	2603±259	ns
N. of retrieved oocytes/OPU	6.1±0.7	6.2±0.9	ns
OSI—FSH/retrieved oocyte (IU)	384±45	434±48	ns
N. of MII oocytes/OPU	5.0±0.8	4.8±0.8	ns
N. of fertilized oocytes/OPU	4.2±0.7	4.0±0.9	ns
N. of transferred embryos/patient	2.3±0.2	2.1±0.1	ns
Embryo morphological score	7.5±2.7	7.4±2.5	ns

DGC-W = density gradient centrifugation and washing.

Data are expressed as mean ± SD

Statistical analysis

The Wilcoxon rank test for unpaired data was used to compare the PLM parameters of oocytes and embryos obtained in CCs with those of oocytes and embryos of NCCs. The multiple regression analysis was performed to evaluate the relationship between PLM parameters and the embryo morphological score.

The GraphPad Prism® software version 4.0, and the STATA® software were used for statistical analysis. A *p* value below 0.05 was considered statistically significant.

Results

In the 86 ICSI cycles included in the study, 258 MII oocytes were inseminated and 209 embryos derived from them were transferred in utero. A clinical pregnancy was obtained in 34 cycles (conception cycles; CCs), whereas the other 52 cycles were unsuccessful (non-conception cycles; NCCs). All pregnancies were singleton, and the overall pregnancy rate per embryo transfer (PR/ET) was 39.5 %.

When CCs and NCCs were compared (Table 1), no significant differences were noticed for the patients' clinical characteristics, the ovarian sensitivity to exogenous FSH

(OSI), the severity of male factor, the number of retrieved oocytes, the number of mature (MII) oocytes, the number of injected oocytes as well as the number and morphological quality (score) of the replaced embryos.

The PLM analysis showed that the average area of the IL-ZP was significantly lower in the oocytes obtained in CCs than in those obtained in NCCs (*p*=0.001; Table 2), whereas the IL-ZP retardance was comparable in CCs and NCCs. Also MS area, major axis length and retardance were similar in the oocytes observed in CCs than in those obtained in NCCs.

The retardance of IL-ZP was remarkably constant during fertilization and cleavage; differently, the IL-ZP area markedly increased from the oocytes to the corresponding embryos (46 % increase in CCs and 20 % increase in NCCs), remaining, however, significantly lower in the embryos transferred in CCs than in those transferred in NCCs (*p*=0.002, Table 2).

The division into *thin-ZP*, *thick-ZP* and *medium-ZP* applied to oocytes (based on IL-ZP area percentiles and not on absolute values) remained practically unvaried when applied to embryos, allowing to divide the transferred embryos into *thin-ZP*, *thick-ZP* and *medium-ZP* embryos that corresponded at 95 % to oocytes belonging to the same subgroup. Considering only patients that received a double embryo

Table 2 Polarized light microscopy data of oocytes and embryos in conception (CCs; *n*=34) and non-conception cycles (NCCs; *n*=52)

	CCs	NCCs	<i>p</i>
N. of oocytes analyzed	102	156	
MS area (μm ²)	197±141	271±179	ns
MS major axis length (μm)	15±8.8	17.5±10.5	ns
MS retardance (nm)	1.7±0.5	1.6±0.6	ns
IL-ZP area—oocytes (μm ²)	6355±741	10073±436	0.001
IL-ZP retardance—oocytes (nm)	1.9±0.6	2.1±0.6	ns
N. of embryos analyzed by PLM	86	123	
IL-ZP area—embryos (μm ²)	9206±911	12060±542	0.002
IL-ZP retardance—embryos (nm)	2.1±0.7	2.2±0.6	ns

Data are expressed as mean ± SD

transfer ($n=50$), we observed that when at least one of the transferred embryos belonged to the *thin-ZP* subgroup (and thus derived from a *thin-ZP* oocyte) the PR/ET was 54.5 % (12/22) and the implantation rate 27.2 % (12/44); differently, when both transferred embryos belonged to either the *thick-ZP* or *medium-ZP* subgroups (thus were obtained from *thick-ZP* or *medium-ZP* oocytes) the PR/ET was only 21.4 % (6/28), with an implantation rate of 10.7 % (6/56).

Finally, the multiple linear regression analysis of PLM data and embryo morphological score showed an inverse, significant correlation between IL-ZP area in embryos and the embryo score ($r=-0.67$, $p=0.011$), whereas the other PLM parameters had no significant relationship with embryo score.

Discussion

The clinical value of the morphological assessment of oocyte competence is still a matter of debate, as among the remarkable number of variables that have been studied [1, 2], none was found to be fully reliable in indicating oocyte competence for further development [3].

Since neither fixation, nor staining of the cell is required, PLM represents one of the few instruments feasible for a non-invasive oocyte assessment [4]. It is still a debated, controversial issue, if PLM can be useful for the identification of good competence oocytes, for their selection and finally for limiting the number of inseminated oocytes maintaining acceptable results.

Some studies suggested that PLM analysis of the MS in fresh and frozen oocytes could be useful in identifying eggs with a high potential of originate embryos leading to pregnancy [5–8]. On the other side, however, the value of MS assessment by PLM was questioned by a study performed by confocal microscopy, that showed that PLM gives only an approximate idea of the real shape and structure of the spindle, as its results do not match with those obtained using fixation, staining and confocal microscopy [14]. Herein we found that oocytes associated with ICSI cycles in which a pregnancy was obtained had a MS mean area, length of the major axis and retardance comparable to those of oocytes obtained in non-conception cycles. In our hands the PLM analysis of the MS, that was performed immediately after cumulus cells removal in controlled conditions (temperature, lighting, etc.), failed to get informations that may help to distinguish an oocyte possibly leading to conception.

Differently, we observed that oocytes with a low value of the IL-ZP area are more frequently able to generate embryos with a high morphological score, and are associated with conception. IL-ZP area increases during the processes of oocyte fertilization and embryo cleavage, but oocytes with a thin IL-ZP have a 95 % chance to originate embryos that still have a thin IL-ZP. We observed that IL-ZP area is

significantly lower both in oocytes and embryos associated with pregnancy than in unsuccessful ICSI cycles. Moreover, when at least one embryo with a low IL-ZP area (that derives from an oocyte with a low IL-ZP area) is transferred, the pregnancy chance results to be approximately double than the one observed when embryos derived from oocytes with a high IL-ZP area are replaced (PR/ET 54.5 % vs. 21.4 %). These findings substantially agree with those of some previous studies suggesting an inverse relationship between IL-ZP thickness and oocyte competence [8–13], but were obtained used IL-ZP area measurement instead of IL-ZP thickness measurement. Indeed IL-ZP area appears to be more reliable and to have a lower intra- and inter-observer variability than IL-ZP thickness, because it is automatically measured by the PLM software system that was used in the present study.

We do not know precisely the reason(s) why having a low IL-ZP area represents a good characteristic for both oocytes and embryos; we can speculate that it could just be a sign of an optimal cytoplasmic maturation of the oocyte during folliculogenesis, or that a high ZP area might affect the correct hatching of the blastocyst and lower the implantation chance. A clear cut-off value identifying the threshold IL-ZP area allowing embryo implantation could not be established in our study; if identified by further studies, it will be of value in order to perform zona thinning or assisted hatching for selected embryos with excessive IL-ZP area.

Another PLM parameter, IL-ZP retardance, was previously proposed as a putative marker of good embryo quality [12]. We could not confirm this finding, as we did not observe any significant difference in the mean IL-ZP retardance between conception and non-conception cycles, both in oocytes and embryos. The discrepancy between our results and those previously reported could depend on the use of a different system for PLM analysis.

In conclusion, our study suggests that PLM can provide and interesting marker of oocyte competence, the area of the inner layer of the ZP. The measurement of IL-ZP area of oocytes is useful to identify oocytes that are likely to originate highly scoring embryos, its measurement in embryos is useful to identify embryos with high chance of implantation.

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