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Is there any correlation between oocyte polarization microscopy findings with embryo time lapse monitoring in ICSI program?

Azita Faramarzi[1] · Mohammad Ali Khalili[1] · Azam Agha-Rahimi[1] · Marjan Omidi[1]

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Abstract
Purpose The aim was to investigate the relationship between the presence of the meiotic spindle (MS) and zona pellucida (ZP) birefringence of MII oocytes with morphokinetics variables of derived embryos in ICSI setting.
Methods Using a polarization imaging system, the ZP birefringence and presence of MS were evaluated pre ICSI. Also, morphokinetics variables including time of second PB extrusion (tPB2), time of pronuclei appearance (tPNa), time of pronuclei fading (tPNf), time of two to eight discrete cells (t2–t8) ECC1 (t2−tPB2), cc2a (t3−t2), S2 (t4−t3) and S3 (t8−t5) as well as irregular cleavage events of 368 embryos were analyzed with time lapse monitoring (TLM).
Results t5 occurred earlier in high birefringent ZP (HB-ZP) compared with low birefringent oocytes (LB-ZP; $p = 0.001$). In addition, t2 happened later in invisible MS compared to visible MS oocytes ($p = 0.013$). There were significantly lower rates of cell fusion (Fu) in oocytes with HB-ZP and also the Fu and trichotomous mitoses (TM) together in visible MS oocytes ($p = 0.005$, $p = 0.001$ and $p = 0.001$, respectively).
Conclusions Both t2 and t5 timings and irregular cleavage events of embryos were correlated with ZP birefringence and MS status, respectively. So, combining the information from both oocyte polarization microscopy imaging and embryo TLM can be a useful tool for single embryo transfer (SET) program.

✉ Mohammad Ali Khalili
Khalili59@hotmail.com

[1] Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Bouali Ave., Safaeyeh, Yazd, Iran

Keywords Oocyte quality · Meiotic spindle · Zona pellucida · Time laps monitoring · Embryo kinetics · Irregular cleavage

Introduction

Currently, multiple pregnancies associated with increased maternal and fetal complications are worrying problem in assisted reproductive technique (ART). Concurrently, elective single embryo transfer (SET) has been suggested for reduction of complications in ART program [1]. Proficiency of the embryo selection with the highest implantation potential is the main role in success of SET [2]. Various methods, such as metabolic profiling, O2 respiration, aneuploidy screening and gene expression survey for human embryo selection have been introduced. However, grading systems based on morphology still remain the preferred way of identifying embryonic development [3, 4].

Extended culture condition and continuous observation of the embryos to the blastocyst stage is one technical approach. Nonetheless, about the perinatal, obstetrical and later outcomes of babies conceived from embryos developed in an extended in vitro culture are still existing concerns [5]. Thus, there is a need for better knowing of early cleavage embryos. Morphokinetics developmental information aids the embryologists to discriminate impalpable timing diversities between developmental stages in embryos with same morphological appearance [6]. Also, conventional morphology assessments performed at discrete time points are subjective. Newly, time lapse monitoring (TLM) has been invented into clinics [7]. Image monitoring by TLM is a safe method that continuously surveys the embryo development and enhances the quality

and quantity of data without disturbing the culture conditions [8].

Oocyte quality, especially optimal cytoplasmic and nuclear maturity is one of the most important variables determining the developmental potential of embryos [9–11]. Moreover, for achieving the best outcomes following SET, many attempts have been done to pinpoint the oocyte parameters that can predict implantation [12]. In an effort to optimize the outcomes of ART treatment, a non-invasive method, the polarization microscopy imaging, for evaluating zona pellucida (ZP) birefringence and meiotic spindles (MS) visualization has been adjudged as predictor factor for oocyte quality. In addition, ICSI with the aid of polarization microscopy became simpler due to knowing the presence and precise location of MS in the oocytes [13]. During ovulation, fertilization and early development, multilaminar glycoprotein layers of ZP, which is constituted of filaments arranged in various orientations, surround the oocyte. The ZP birefringence status may have influential role on oocyte quality [14]. Dynamic constitution of MS made by microtubules has various functions that are vital for fertilization process [13].

The purpose of this prospective study was to assess the relationship between the oocyte ZP birefringence, MS and morphokinetics variables as well as the cleavage patterns of embryos in clinical setting.

Materials and methods

Study design

A total of 478 MII oocytes were retrieved from 54 women aged 31.0 ± 3.42 years undergoing ICSI cycles between August 2015 and August 2016. Cycles with severe male factor (sperm concentration $<4 \times 10^6$/ml), frozen and surgically retrieved spermatozoa were eliminated due to poor recovery of motile spermatozoa. Study inclusion criteria were as follows: patients with fresh autologous oocytes and at least one normal fertilized oocytes. Ethics committee of our institution approved this study and informed consent was obtained from each participant.

Ovarian stimulation and oocyte preparation

The patients were stimulated with the standard GnRH antagonist protocols. In the antagonist protocol, 150 IU/day of follicle-stimulating hormone (Gonal F, Serono, Geneva, Switzerland) was administered on day two of the menstrual cycle. When at least one follicle reached 14 mm, 0.25 mg of a GnRH antagonist (Cetrotide, Merck Serono, Darmstadt, Germany) was initiated and continued until the day of human chorionic gonadotropin (hCG) injection. When proper follicular development was viewed on transvaginal ultrasound, recombinant hCG (rhCG; Merck Serono, Darmstadt, Germany) was administered to trigger final maturation and ovulation. 36 h later, ultrasound-guided oocyte collection was performed using a single lumen aspiration needle (Wallace; Smiths Medical International, UK). Retrieved cumulus–oocyte complexes (COCs) were incubated in culture medium (G-IVF; Vitrolife, Kungsbacka, Sweden) covered with mineral oil (Ovoil; Vitrolife) at 37 °C and 6% CO2 for 2–3 h. The COCs were denuded of their cumulus and coronal cells by 30–60 s exposure to HEPES buffered medium containing 80 IUmL/1 hyaluronidase (Irvine Scientific, CA, USA) and by pipetting the COCs. MII oocytes were injected using husbands prepared sperm. ICSI was then performed as described previously [13].

Imaging of the ZP and MS

Immediately before ICSI, approximately 38–40 h after hCG triggering, ZP birefringence and MS visualization were assessed. Each of 478 MII oocytes was put in a 3 μL droplet of buffered medium (G-Mops-V1; Vitrolife) in a glass-bottomed culture dish (WillCo-Dish; Bellco Glass NJ, USA) covered with previously equilibrated mineral oil. ZP imaging and visualization of the MS was done on Nikon TE-300 inverted microscope. Analysis of birefringence, including autocalibration, was controlled by a polarization imaging software module (OCTAX ICSI Guard™, Microscience, Germany) done with an imaging software system (OCTAX Eyeware™). Imaging software record combined with bright field (green) and birefringence (red) visions for each oocyte. Oocytes were categorized into low birefringent ZP (LB-ZB) or high birefringent ZP (HB-ZP), based on uniformity and intensity of the inner ZP birefringence. HB-ZP oocytes had high-intensity birefringent inner ZP layers. It was mostly uniform around the oocyte as well as equally bright and many thick ZP inner layers. LB-ZB oocytes were defined as oocytes showing low or uneven birefringence distribution within inner layer of ZP (Fig. 1). All classifications were done by one embryologist [15].

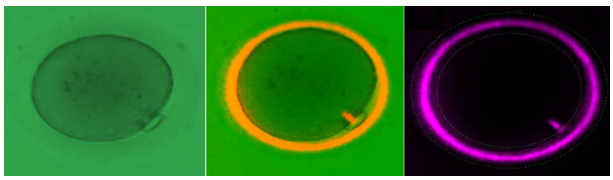

Fig. 1 Polarization microscopy (OCTAX PolarAID; Octax, Herbon, Germany) image of a human oocyte. A bright meiotic spindle is clearly visible and HB-ZP

Embryo culture

After ICSI, each oocyte was individually placed in a pre-equilibrated culture slide TLM system (Primo Vision Time-lapse Embryo Monitoring System, Goteborg, Sweden). The Primo Vision dish with a central depression containing nine straight-sided circular wells was filled with 40 µl of G-1+ medium, covered by 3 ml of mineral oil. The culture slides were pre-equilibrated (37 °C and 6% CO2) using fresh G-1+ medium and mineral oil. The culture slide was placed in a time lapse microscope at 37 °C, 5% O2 and 6% CO2, immediately after ICSI for 3 days.

Time-lapse imaging system

The camera of Primo Vision was set to take pictures of each embryo every 10 min and the total scan of embryos was taken every 20 min. On day 3, embryos were morphologically assessed for timing of cell divisions and development. The following early kinetic markers were evaluated from 368 normally fertilized oocytes: time of second PB extrusion (tPB2), time of pronuclei appearance (tPNa), time of pronuclei fading (tPNf), time of two to eight discrete cells (t2−t8), duration of first cell cycle (ECC1 = t2−tPB2), duration of second embryo cell cycle (cc2a = t3−t2), complete second synchronous divisions s2 (t4−t3), the time to complete third synchronous divisions and synchronization of cleavage pattern s3 (t8−t5).

Three cleavage anomalies specifically monitored were: cell fusion (Fu), where fusion or merging of blastomeres causes reduction in embryo cells number which gives the reversed cleavage appearance to embryos. In the other words, a blastomere was reabsorbed after cleavage. Trichotomous mitoses (TM), where a single blastomere divided directly from 1 to 3 daughter cells [16] (Fig. 2).

Data analysis

Results are expressed as mean ± standard deviation (SD) for normal numeric variables, median ± interquartile range and percentage for categorical variables. The means of samples with normal distribution and of sufficient size were compared by Independent Samples *T* test. In case of non-normal distribution, the medians were compared by Mann–Whitney. Categorical variables were compared using Chi-square. p value <0.05 was considered as statistically significant. The analysis was performed using the Statistical Package for the Social Sciences 20 (SPSS Inc., Chicago, IL, USA).

Results

A total of 590 COCs were retrieved, of which 21 (3.56%) were discarded after denudation because of an abnormal appearance (e.g., extensive vacuolization or degeneration). Of the remaining, 478 (∼84%) MII oocytes were injected and 368 (∼77%) of them fertilized normally. Nine zygotes were excluded from further analysis due to arresting in early development stages and/or excessive fragmentation.

Correlation of ZP birefringence and MS visualization with morphokinetics variables

TLM showed that there was no significant relationship between HB-ZP and early developmental events of tPB2, tPNa, tPNf, t2, ECC1, t3, CC2a, t4, S2, t6, t7, t8 and S3 ($p > 0.05$). However, t5 occurred earlier in oocytes with HB-ZP compared to LB-ZP ones ($p = 0.001$; Table 1). According to Table 2, visualized MS oocytes or non-visualized MS oocytes had morphokinetics variables (tPB2, tPNa, tPNf, t2, ECC1, t3, CC2a, t4, S2, t5, t6, t7, t8 and S3)

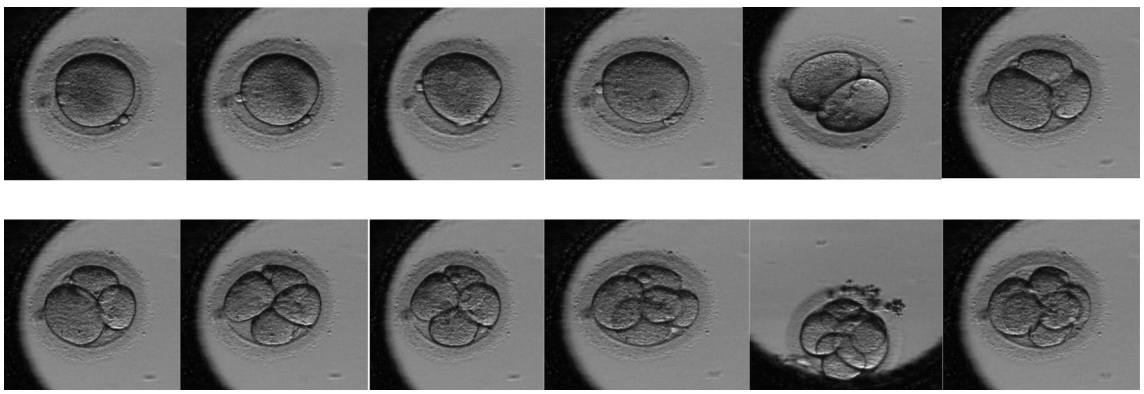

Fig. 2 Time lapse monitoring (Primo Vision Time-lapse Embryo Monitoring System, Goteborg, Sweden) of embryo derived from oocyte with HB-ZP and visible MS

Table 1 Correlation of ZP birefringence and Spindle visualization with morphokinetics variables

Morphokinetics variables	ZP birefringence		*p* value	Spindle visualization		*p* value
	High	Low		Visible	Not visible	
tPB2 (368)	2.21 ± 0.40	2.28 ± 1.04	0.254	2.28 ± 0.59	2.21 ± 0.25	0.978
tPNa (368)	8.01 ± 2.33	8.15 ± 3.01	0.119	7.53 ± 2.00	8.21 ± 2.30	0.061
tPNf (368)	21.13 ± 2.57	21.03 ± 4.48	0.987	21.08 ± 3.25	21.08 ± 3.06	0.117
EEC1 (368)	2.30 ± 1.38	2.15 ± 1.28	0.202	2.40 ± 1.16	2.10 ± 0.30	0.247
t2 (365)	24.03 ± 3.01	23.15 ± 6.01	0.311	25.11 ± 5.03	26.09 ± 5.06	0.013
t3 (365)	35.06 ± 5.19	35.39 ± 6.19	0.236	35.22 ± 4.50	35.30 ± 5.51	0.499
CC2a (365)	12.50 ± 8.35	10.05 ± 7.02	0.680	11.25 ± 9.10	13.20 ± 8.11	0.712
t4 (364)	40.55 ± 4.24	39.11 ± 6.58	0.644	41.05 ± 4.00	40.30 ± 5.09	0.343
S2 (364)	4.15 ± 8.23	4.54 ± 5.40	0.068	6.20 ± 7.39	5.20 ± 9.10	0.549
t5 (363)	49.31 ± 8.06	53.39 ± 9.17	0.001	50.30 ± 8.10	50.51 ± 9.12	0.544
t6 (363)	54.17 ± 7.15	55.10 ± 7.27	0.234	54.51 ± 7.07	54.04 ± 7.49	0.350
t7 (361)	53.40 ± 6.00	53.28 ± 4.51	0.249	53.16 ± 5.11	54.34 ± 7.11	0.517
t8 (359)	61.29 ± 6.44	60.17 ± 5.41	0.278	61.06 ± 6.54	62.12 ± 6.36	0.365
S3 (359)	13.33 ± 5.47	13.02 ± 5.49	0.451	13.25 ± 5.51	13.09 ± 5.41	0.581

Results are expressed as mean ± standard deviation (SD) for normal numeric variables, median ± interquartile range and percentage for categorical variables. The second polar body is completely separated from the oolemma (tPB2), pronuclei appearance (tPNa), pronuclei fading (PNf), t2 = first cleavage (2-cell stage); t3 = second cleavage (3-cell stage); t4 = 4-cell stage; t5 = 5-cell stage; t6 = 6-cell stage; t7 = 7-cell stage t8 = 8- cell stage. Duration of first cycle cell (ECC1: t2-PNf), durations of the second cycle (cc2a; t3-t2), the time to complete second synchronous divisions (s2:t4-t3) and the time to complete third synchronous divisions (s3:t8-t5)

Values in parenthesis are the embryo number

Table 2 Compression of uneven blastomeres between zygotes derived from HB-ZP and LB-ZP oocytes also visible and not visible MS oocytes

Variables		Uneven blastomeres		OR	*p* value
		Yes	No		
ZP birefringence	High	27	247	1.175 (0.514–2.685)	0.702
		77.1%	74.2%		
	Low	8	86		
		22.9%	25.8%		
Spindle visualization	Visible	15	153	0.882 (0.437–1.783)	0.727
		42.9%	45.9%		
	Not visible	20	180		
		57.1%	54.1%		

ZP zona pellucida, *OR* odds ratio, *CI* confidence interval

($p > 0.05$). However, t2 occurred earlier in the oocytes with visible spindle ($p = 0.013$; Table 1).

Correlation of ZP birefringence and MS visualization with uneven blastomeres embryos

The data showed that there was no significant relationship between ZP birefringence, MS visualization and the rate of embryos with uneven blastomeres (77.1 vs 74.2% and 42.9 vs 54.1%, respectively) (Table 2).

Correlation of ZP birefringence and MS visualization with Fu embryos

There was significant relationship between ZP birefringence and rate of Fu embryos (37.4 vs 62.6%; $p = 0.005$). Also, there was significant relationship between spindle visualization and reverse cleavage rate (32.3 vs 67.7%; $p = 0.001$) (Table 3).

Table 3 Correlation between ZP birefringence, spindle visualization and rate of cell fusion (Fu) embryos

Variables		Fu		OR	*p* value
		Yes	No		
ZP birefringence	High	101	169	0.492 (0.305–0.793)	0.005
		37.4%	54.8%		
	Low	51	42		
		62.6%	45.2%		
Spindle visualization	Visible	54	113	0.478 (0.311–0.733)	0.001
		32.3%	50.0%		
	Not visible	98	98		
		67.7%	50.0%		

ZP zona pellucida, *Fu* cell fusion, *OR* odds ratio, *CI* confidence interval

Correlation of ZP birefringence and MS visualization with TM

The findings also showed that there was insignificant correlation between ZP birefringence and rate of TM embryos (66.7 vs 75.8%; $p = 0.127$); but as presented in Table 4, there was significant relationship between spindle visualization and TM (24.2 vs 51.2%; $p = 0.001$).

Discussion

One of the challenges in successful ART practice is the selection of embryos with the highest developmental competence [17]. The search for a non-invasive method of embryo assessments with the highest competence to produce a live birth continues to disappoint the ART clinicians [18]. Lately, evaluating of ZP birefringence and MS imaging has been considered as a promising approach for oocyte quality assessments [13]. Various studies surveyed correlation between oocyte quality and embryo outcome by conventional assessment [13, 19–23].

However, there is only study reporting the noninvasive imaging systems of polarization microscopy and TLM for assessment of oocyte quality and embryo morphokinetics analysis in one setting. Kobayashi et al. showed that in women with advanced age (>39 years), oocytes MS were not related to embryo morphokinetics. However, embryo arrest before 8 cell stage was higher in oocytes with abnormal MS [24]. In contrast, we included young patients with 31.0 ± 3.42 years in our study.

TLM is an emerging tool which pinpoints the parameters that can foretell the developmental potential of cleaving embryos under stable development conditions [3]. With the aid of continuous TLM, the present study aimed to assess the ZP birefringence and MS visualization as oocyte quality and its influence on embryo morphokinetics parameters and cleavage patterns. The data showed that oocytes HB-ZP and visible spindle displayed shorter t5 and t2 compared with LB-ZP and invisible spindle oocytes. However, ZP birefringence and spindle visualization did not influence other morphokinetics time points.

Recently, it was reported that the presence of a normal spindle is one of the factor affecting the suitable attainment of nuclear and cytoplasmic oocyte maturity [25]. Also, fertilized oocytes with abnormal or visible MS have a lower capacity to continue with normal embryos [19] that concur with our results. However, other reported that the

Table 4 Correlation between ZP birefringence, spindle visualization and rate of trichotomous mitosis (^{TM}) embryos

Variables		TM		OR	*p* value
		Yes	No		
ZP birefringence	High	44	222	0.640 (0.359–1.139)	0.127
		66.7%	75.8%		
	Low	22	71		
		33.3%	24.2%		
Spindle visualization	Visible	16	150	0.305 (0.166–0.560)	0.001
		24.2%	51.2%		
	Not visible	50	143		
		75.8%	48.8%		

ZP zona pellucida, *TM* trichotomous mitosis, *OR* odds ratio, *CI* confidence interval

visualization of the MS displayed no discrepancy in the implantation or pregnancy rates [21]. Multiple reasons exist for this inconsistency, involving differences in response of the patients to the ovarian stimulation, the timing of oocyte observation, oocyte handling and insemination method [26].

The MS has main role in physiology of the oocyte influencing the embryo development. MS changes may induce alteration in the chromosome complement, impelling nondisjunction aneuploidy, unbalanced chromatid junction, as well as lack of chromosomes that affect the embryo competence [25]. According to Ebner and associates, there are positive relationship between ZP birefringence and embryo development [23]. However, others found that ZP and MS visualization cannot be a true predictor of the ART outcomes [27]. Regular constitute of ZP may reflect favorable oocyte cytoplasmic potential. Thus, HB-ZP oocytes have the optimal competence potential for embryo development and implantation [19]. ZP has various roles in both fertilization procedure and embryo development. Sperm must attach to the ZP, impel acrosome reaction, pierce the ZP and combine to the oolemma. Cortical granules releasing after fertilization alter ZP structure, so polyspermy will be prevented. In addition, ZP protects the loose contact blastomeres during cleavage stage [28].

Since the advent of TLM, improved pregnancy outcomes have been reported via uninterrupted culture and continuous monitoring of human embryos. Selection of embryos with high implantation potential by morphokinetics analysis relies greatly on the timing of cell divisions [29]. However, Lemmen and colleagues showed that early cleavage timing was positively related to the embryo developmental potential [30]. The first cleavage or t2 timing has been surveyed widely and is accepted as a good parameter of embryo development, and early cleavage preferable parameters compared with late cleavage [31]. Meseguer and co-workers also proposed a hierarchical embryo selection model based on: first criteria: timing of t5; second criteria: S2; and third criteria: CC2. On base it, implantation rate and blastocyst formation increased considerably [31]. Also, it is generally accepted that early cleavage is a good indicator of developmental competence [32]. Several elucidation have been proposed about main mechanisms between t2 and derived embryos: highly regulated process responsible convey one fertilized oocyte to two cells embryo that begin with temporary increment in intracellular Ca^{2+}, integrity in DNA replication, maturation of oocyte and intrinsic properties of gametes, such as HLA-G [33]. Moreover, our results showed that the rate of cell fusion was higher in LB-ZP oocytes as well as FU and Trichotomous mitosis rates were higher in oocytes with undetectable spindles. Beside kinetic parameters, there are different events seen with TLM that may help for selection of the best embryos in SET. The imaging of abnormal embryo cleavages, such as reverse cleavage is not possible to detect with conventional microscopy [34]. Genetic disorders and aneuploidies are the possible reasons for embryos to avoid from normal cleavage pattern. Also, it was shown that multiple aneuploidies exist in the majority of embryos that do not follow normal cell divisions timing [31].

Some studies demonstrated that cleavage pattern, such as embryo with uneven blastomere size at 2-cell stage, direct cleavage and reverse cleavage resulted in lower developmental potential and implantation [6, 31, 35]. TLM reveals abnormalities of embryo division correlated to reduce implantation potential. The molecular mechanisms that cause abnormal cleavage are not obvious yet. It seems rational to suppose that mitotic errors may play a main role in this phenomenon [36]. Liu and colleagues also showed that RC related factors are not limited to those affecting the oocyte, but also include sperm poor motility, which is not repaired by poor quality oocytes [6].

The present study focused on the ZP birefringence and MS presence of the oocytes and their relationship with morphokinetics parameters and atypical cleavage with the aid of TLM. The limitations of this study were related to small sample size plus lack of data on the implantation and pregnancy outcomes. Therefore, future studies are warranted to validate findings of the present study. In conclusion, the t2 and t5, as well as cleavage pattern of embryos were correlated with ZP birefringence and MS status. Moreover, the impact of these related data is severely limited due to very heterogeneous and combined phenomena of different origin. Nevertheless, it seems that embryo selection based on data collected from polarization microscopy imaging can significantly ameliorate good morphokinetics embryos with high developmental potential for SET program.

Compliance with ethical standards

Funding This study was funded by Yazd research and clinical center for infertility.

Conflict of interest All authors declare no conflict of interest.

Ethical approval The present study conforms to the provisions of the Declaration of Helsinki. Also, this study was approved by the Institutional Review Board of the Yazd Research and Clinical Center for Infertility (IR.SSU.MEDICINE.REC.1395.2). The unique nature of identifying patients was completely preserved.

Informed consent Informed consent was obtained from all participants included in the study.

Authors' contribution Azita Faramarzi: data collection, methodology, writing; Azam Agh-Rahimi: writing, data analysis; Marjan Omidi: language editing, data analysis; Mohammad Ali Khalili: leader of study, methodology, editing the paper.

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