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# Noninvasive assays of in vitro matured human oocytes showed insignificant correlation with fertilization and embryo development

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**Abstract**
*Purpose* Recently, the upgrading of in vitro maturation (IVM) of human oocytes as a promising strategy has emerged in assisted reproductive technology (ART). The goal was to evaluate the correlation of the in vitro matured oocytes selected on the basis of the zona pellucida (ZP) birefringence and meiotic spindles (MS) detection with fertilization and subsequent embryo development in ICSI program.
*Methods* A total of 168 immature oocytes [germinal vesicle ($n = 140$) and metaphase I ($n = 28$)] obtained from patients undergoing oocytes retrieval for ICSI. After in vitro culture for 24–40 h, 112 (67 %) oocytes reached to MII stage. Using a polarized microscopy, the presence of MS and ZP birefringence were assessed in matured oocytes, followed by ICSI performance.
*Results* The rates of fertilization in oocytes with spindles (51.3 %) were similar to that of the oocytes without spindles (50.7 %; $P = 1.00$). Moreover, the fertilization rates in high birefringence (HB) oocytes was not statistically different than oocytes with low birefringence (LB) ($P = 0.44$). The findings also showed that 64.9 % of the fertilized oocytes developed to embryos, in which 33.3 % were derived from spindle-detected oocytes. Regarding the ZP birefringence, 35.5 % of the embryos were derived from HB oocytes.
*Conclusions* There were insignificant relationships between the MS detection and ZP birefringence score with the rates of fertilization and embryo development in IVM oocytes.

**Keywords** IVM · ZP birefringence · Meiotic spindles · Fertilization · Embryo development

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## Introduction

Recently, in vitro maturation (IVM) of oocytes has emerged as a promising strategy in assisted reproductive technology (ART). Retrieval of immature oocytes from unstimulated ovaries, followed by IVM has been proposed to avoid the side effects of exogenous gonadotropin administration. In this technology, the ovarian hyperstimulation syndrome (OHSS) is noticeably suppressed by reduction or elimination of gonadotropin stimulation. Also, IVM program is useful for infertile patients with polycystic ovarian syndrome (PCOS) [1–4].

The indications for IVM have been now expanded to other fields, such as fertility preservation, for young women undergoing anticancer therapy of radiation and/or chemotherapy [5, 6]. Moreover, it is recommended that immature oocytes undergo IVM procedure immediately or cryopreserved, if it becomes necessary [7]. Meanwhile, the oocytes maturation subsequent to IVM, rarely lead to successful pregnancy and implantation [8]. Many studies reported that IVM might impose adverse effects on oocytes spindles and chromosome organization, which is associated

with lower developmental capability of the embryos derived from IVM program [9]. Many reports have proven that oocyte quality is associated with implantation potential of derived embryos [10, 11]. Advances in assisted conception necessitated noninvasive markers to select the best quality oocytes as the predictor of the finest embryos [3]. Oocyte quality is mainly based on extracellular and intracellular morphological characteristics evaluated with inverted light microscopy [12]. However, it is still complicated to predict or select the metaphase II oocytes resulting in embryos with high implantation potential [13].

Recently, a promising approach for assessing zona pellucida (ZP) birefringence and meiotic spindles (MS) visualization has been considered as prognostic marker for oocyte quality [3]. A quick and noninvasive visualization of the birefringent structures, such as MS and ZP becomes possible without any previous preparation. Also, with the aid of this technology, intracytoplasmic sperm injection (ICSI) procedure became easier due to the exact awareness of the presence and location of MS in mature oocytes [14]. MS is a dynamic structure composed of microtubules involved in different functions that are crucial for fertilization events [15]. In addition, MS structure may reflect oocyte quality by serving as a marker for cytoplasmic maturation and pH or temperature fluctuations during handling [16]. The ZP is a unique extracellular coat composed of filaments organized in different orientations surrounding the maturing oocytes during ovulation, fertilization and early embryonic development [17]. Therefore, the present study was designed to assess the association between selection of IVM oocytes on the basis of the ZP birefringence and MS detection, with fertilization and subsequent embryo development in ICSI program.

## Materials and methods

### Source of oocytes

A total of 168 immature oocytes from 46 consenting patients undergoing oocyte retrieval for ICSI were included in this prospective study. Among the immature oocytes, 140 were seen in the germinal vesicle (GV) stage and 28 recognized as metaphase I (MI) oocytes. Approval was obtained from ethics committee of Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences. The indications for assisted reproduction consisted of male factor ($n = 32$), polycystic ovary disease ($n = 6$), endometriosis ($n = 4$), and idiopathic ($n = 4$). Controlled ovarian hyperstimulation (COH) was performed as previously described [18]. Oocyte pick-up was scheduled 34–36 h after hCG administration under transvaginal ultrasound guidance.

### Ovarian stimulation and oocyte collection

Ovarian stimulation was achieved by long pituitary down regulation using a combination of a gonadotrophin-releasing hormone (GnRH) agonist or antagonist and FSH (Gonal-F; Serono, Geneva, Switzerland). The recombinant hCG (rhCG; IBSA Co, Switzerland) was administered to trigger final maturation and ovulation, when the ovarian follicles reached 18–20 mm diameter. Thirty-six hours later, ultrasound-guided oocyte collection was performed using a 16-gauge single-lumen aspiration needle (Wallace; Smiths Medical International, UK) with 150 mmHg pressure.

### Oocyte preparation and IVM

After cumulus–oocyte complex (COC) retrieval, the COCs were incubated in culture medium (GIVF, Vitrolife co., Sweden) for 2–3 h. The cumulus cells were then removed by 30–60 s exposure to HEPES buffered medium containing 80 IU $mL^{-1}$ hyaluronidase (Irvine Scientific, CA, USA), and by pipetting the COCs with a pasture pipette. Denuded oocytes were assessed for nuclear maturation stage. Oocytes lacking first polar body (PB) extrusion were considered immature and candidate for IVM. In IVM procedure, immature oocytes were washed in 3 drops of washing medium (SAGE IVF), then incubated in maturation medium (SAGE IVF) supplemented with 75 mIU/mL FSH and 75 mIU/mL LH (Ferring) at 37 °C and 5 % $CO_2$. After 24–48 h, the oocytes were screened for the presence of the first PB by stereomicroscope (Olympus, Tokyo, Japan) to determine maturity.

### Meiotic spindles detection, ZP examination and ICSI

Oocyte imaging and ICSI was performed at the same time. For this purpose, mature oocytes were placed individually in 4-μL droplet of buffered medium (G-Mops-V1; Vitrolife co., Sweden) in a glass-bottomed culture dish (WillCo-Dish; Bellco Glass NJ, USA) covered with warm mineral oil (Irvine Scientific). Prepared sperm samples using density gradient technique [19] were placed in a central droplet of polyvinylpyrrolidone (PVP) solution (Irvine Scientific, CA). The oocytes were imaged under an inverted microscope (TE300; Nikon, Tokyo, Japan) mobilized with a stage heated to 37 °C and a polarizing optical system (OCTAX PolarAIDE; Octax). This system reveals birefringent structures of ZP and MS, using OCTAX Eyeware software. ZP scoring was automatic and oocytes were classified as having a high (HB) or low (LB) birefringent ZP. For oocytes with detected spindles, ICSI was performed after the spindles were placed at 6 or 12 o'clock position. For oocytes without birefringent spindles, ICSI

was performed after placing the first PB at 6 or 12 o'clock position. The injected oocytes were washed twice and cultured in droplets of G1 (Vitrolife co., Sweden) for subsequent analysis.

### Fertilization and embryo assessment

Fertilization was checked after 16–18 h post-ICSI. Normally, the oocytes with two pronuclei (2PN) and a second PB were judged as sign of fertilization. In the next step, fertilized oocytes were washed twice and cultured in fresh cleavage media (G1; Vitrolife co., Sweden). On day 2, embryo cleavage was evaluated according to the laboratory protocols.

### Statistical analysis

Data are presented mean ± SD and odds ratios (OR) with 95 % confidence intervals (CI), as appropriate. Data expressed as percentages were compared by the Chi-squared and Fisher's exact tests, wherever appropriate. Results were analyzed using SPSS version 18 (SPSS, Chicago, IL, USA). Probability of $P < 0.05$ was considered as statistically significant.

## Results

The overall number of collected oocytes from 103 ICSI cycles were 1,284; of which 168 (13 %) were classified as immature: 140 (10.9 %) at GV and 28 (2.18 %) at MI stages. The mean female age was 30.47 ± 4.6 years. After in vitro culture for 24–48 h, 112 (67 %) of the oocytes reached to MII stage. Ninety (64.3 %) GV and 22 (78.6 %) MI oocytes were matured after IVM ($P = 0.18$). Among matured oocytes, the rates of oocytes with spindles were lower than oocytes without spindles (34.8 vs. 65.2 %); whereas, the rates of HB oocytes were higher than the LB oocytes (55.4 vs. 44.6 %).

After ICSI, the rate of fertilization achieved by IVM oocytes was 50.9 %. As shown in Table 1, the data also revealed that 51.3 % of the oocytes with detected spindles were fertilized normally, forming 2 pronuclei and releasing the second PB, compared with the oocytes without spindles (50.7 %; $P = 1.00$). Moreover, in ZP birefringence category, the fertilization rate in HB oocytes was 54.8 vs. 46 % in LB oocytes ($P = 0.44$; Table 2). The findings also confirmed that 37 out of 57 (64.9 %) of the fertilized oocytes following IVM reached to embryo formation, in which 13 (33.3 %) were derived from spindle-detected oocytes. Regarding the ZP birefringence, 22 (35.5 %) of the embryos were derived from HB oocytes.

In subanalysis, the fertilization rate and embryo development were compared in matured oocytes after IVM application regarding two categories: time of maturation (24 vs. 48 h, Table 3), and stage of immaturity (GV vs. MI, Table 4). As presented in Table 4, both fertilization and embryo development rates were higher in matured MI than matured GV oocytes ($P = 0.33$ and $P = 0.44$, respectively).

## Discussion

Denudation of the cumulus and corona cells prior to ICSI reveal that approximately 15–20 % of the retrieved oocytes in COH protocols are immature [20, 21]. However, there are still controversies about the safety of IVM, even though some cases of successful fertilization, embryo development, and pregnancy have been reported using clinical IVM [22, 23]. But, in aforementioned studies, the immature oocytes derived from ICSI cycles were usually discarded. So, the present study aimed to assess the oocyte ZP birefringence condition and MS visualization, as well as its correlation with fertilization and embryo development in ICSI program. One study has stated that human oocytes matured in vitro do not have the same developmental potential as those recovered mature from the ovaries [24]. Li and colleagues [9] indicated that IVM may have deleterious effects on the MS and chromosome organization of the immature human oocytes. Moreover, Omidi et al. [3] have recently reported a lower rate of spindles visualization in IVM oocytes than in vivo matured oocytes. Regarding the oocyte ultrastructural assessment, high abnormalities after IVM were detected when compared with in vivo matured oocytes [25]. However, in the present study, the percentage of oocytes with detected spindles was lower among the matured oocytes. It was suggested that certain

**Table 1** Fertilization rate and embryo development in in vitro matured oocytes according to meiotic spindle detection

| Polscope visualization | With spindle | Without spindle | Odds ratio (95 % CI) | *P* value |
|---|---|---|---|---|
| No. of oocytes (%) | 39 (34.8) | 73 (65.2) | | |
| No. of fertilized oocytes (%) | 20 (51.3) | 37 (50.7) | 1.02 (0.47–2.22) | 1.00 |
| No. of embryos (%) | 13 (33.3) | 24 (32.9) | 1.02 (0.44–2.33) | 1.00 |

**Table 2** Fertilization rate and embryo development in in vitro matured oocytes according to the ZP birefringence

| Polscope visualization | High ZP birefringence | Low ZP birefringence | Odds ratio (95 %CI) | *P* value |
|---|---|---|---|---|
| No. of oocytes (%) | 62 (55.4) | 50 (44.6) | | |
| No. of fertilized oocytes (%) | 34 (54.8) | 23 (46) | 0.70 (0.33–1.48) | 0.44 |
| No. of embryos (%) | 22 (35.5) | 15 (30) | 0.77 (0.35–1.73) | 0.55 |

**Table 3** Polscope assessments, fertilization rate and embryo development in 24- and 48-h matured oocytes

| Time intervals | Spindle detection | | ZP birefringence | | Fertilization rate | Embryo development |
|---|---|---|---|---|---|---|
| | Yes | No | Low | High | | |
| 24-h matured oocytes | 33 (35.9) | 59 (64.1) | 41 (44.6) | 51 (55.4) | 46 (50) | 34.8 (32) |
| 48-h matured oocytes | 7 (35) | 13 (65) | 11 (55) | 9 (45) | 11 (55) | 7 (35) |
| Odds ratio (95 % CI) | 1.03 (0.37–2.86) | | 0.65 (0.24–1.73) | | 0.81 (0.31–2.16) | 0.99 (0.35–2.73) |
| *P* value | 1.00 | | 0.46 | | 0.80 | 1.00 |

The values inside parenthesis represent as percentage

**Table 4** Polscope assessments, fertilization rate and embryo development in matured GV oocytes compared with matured MI oocytes

| Maturity status | Spindle detection | | ZP birefringence | | Fertilization rate | Embryo development |
|---|---|---|---|---|---|---|
| | Yes | No | Low | High | | |
| Matured GV oocytes ($n = 90$) | 34 (37.8) | 56(62.2) | 38 (42.9) | 52 (57.1) | 44 (48.4) | 30 (33) |
| Matured MI oocytes ($n = 22$) | 7 (28.6) | 15 (71.4) | 14 (61.9) | 8 (28.1) | 13 (61.9) | 9 (42.9) |
| Odds ratio (95 %CI) | 1.49 (0.52–4.20) | | 0.46 (0.17–1.22) | | 0.57 (0.21–1.52) | 0.65 (0.24–1.72) |
| *P* value | 0.31 | | 0.91 | | 0.33 | 0.44 |

The values inside parenthesis represent as percentage

environmental changes, such as temperature can induce microtubule depolymerization causing subsequent spindles damage [16].

Madaschi and colleagues [15] found that when the MS was not visualized in in vivo matured oocytes, the fertilization rate would reduce significantly. Whereas, our data did not show significant differences in the rates of fertilization between two groups of oocytes in regards to spindles detection. Wang and Keefe [26] imaged the IVM living oocytes with a Polscope for detecting the MS, followed by fixation and evaluation using conventional microscopy. They observed that all oocytes without spindles imaging showed abnormal or no spindles after fluorescent staining. In the present report, we still observed a 50.7 % fertilization rate in the oocytes in which spindles were invisible. Therefore, as others suggested, in vitro manipulation procedures may have induced a temporary spindles disassembly in these oocytes [26].

In our study, there was no difference in the embryo development to day 3 in oocytes with detected spindles compared with oocytes without spindles, which is in accordance with Cohen and associates [13]. However, more development in the oocytes with spindles has been presented by Wang and Keefe [26]. Although, the MS birefringence is an inherent physical property in microtubules detected with the aid of Polscope, unvisualized spindles in the oocytes may be due to the condition of IVM or subsequent development. Furthermore, oocyte aging, maternal age and other patient-dependent factors may disrupt spindles configuration. In addition, the rates of fertilization and embryo development in 24- and 48-h matured oocytes were insignificant, as well as GV and MI maturation rates. One probable reason for these results may be due to noticeable differences in the sample size between the two groups of oocytes.

As, it has been demonstrated previously [3], the percentage of HB was greater than LB in the ZP of in vitro matured oocytes. Despite the fact that different developmental stages and culture conditions may alter the ZP architecture [27], our observation conflicted with

aforementioned study. Moreover, the data generated from this study showed that there was an insignificant tendency for more fertilization and embryo development in oocytes with high ZP birefringence. In parallel with our findings, Braga et al. [27] reported that among immature oocytes, ZP birefringence had no effect on subsequent fertilization and embryo quality. On the other hand, others reported that embryo development was superior in embryos derived from high ZP birefringence oocytes [28]. Since, ZP with HB appears to be an indicator of high oocyte quality [3], the embryos derived from such oocytes may have more developmental potential. Although, we did not find any significant relationship between the MS detection and ZP birefringence score with subsequent fertilization and embryo development in IVM program, further controlled studies with larger sample sizes are necessary to reach a final conclusion.

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**Conflict of interest** None.

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