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Zona pellucida birefringence and meiotic spindle visualisation of human oocytes are not influenced by IVM technology

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Abstract. The aim of the present study was to investigate the relationship between the presence of the meiotic spindle and zona pellucida (ZP) birefringence with morphology of *in vivo*- and *in vitro*-matured human oocytes. Germinal vesicles ($n = 47$) and MI ($n = 38$) oocytes obtained from stimulated ovaries of patients undergoing intracytoplasmic sperm injection (ICSI) underwent IVM. Using a PolScope (OCTAX PolarAID; Octax, Herbon, Germany), the presence of spindles and ZP birefringence was assessed in both *in vivo*-matured ($n = 56$) and IVM ($n = 56$) oocytes. In addition, the morphology of each matured oocyte was evaluated microscopically. There were insignificant differences for ZP birefringence and meiotic spindle between the *in vivo*-matured and IVM MII oocytes. Subanalysis revealed that the rates of morphologically abnormal oocytes did not differ significantly between the two groups, except in the case of irregular shape ($P = 0.001$), refractile body ($P = 0.001$) and fragmented polar body ($P = 0.03$), which were higher in IVM oocytes. In the case of *in vivo*-matured oocytes, a significantly higher percentage of oocytes with intracytoplasmic and both intra- and extracytoplasmic abnormalities have a low birefringent ZP ($P = 0.007$ and $P = 0.02$, respectively). There was no relationship between morphological abnormalities and spindle detection. The findings suggest that clinical IVM is a safe technology that maintains the high maturation rate and integrity of oocytes. In addition, the use of the non-invasive PolScope is recommended for the detection of oocytes most suitable for ICSI.

Additional keywords: oocyte morphology, PolScope.

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Introduction

Following ovarian hyperstimulation, oocytes are retrieved at different stages of nuclear maturation (Cha and Chian 1998). Approximately, 15% of oocytes are immature (i.e. at the germinal vesicle (GV) or MI stages; Nazari *et al.* 2011; Halvaei *et al.* 2012). The IVM of such immature oocytes can be a way to increase the number of embryos, especially in poor responders (Strassburger *et al.* 2004), although pregnancy and implantation rates have been reported to be very low with IVM technology (Halvaei *et al.* 2012). Because ovarian stimulation with large doses of gonadotropins is unnecessary for IVM, the technique is useful for women with polycystic ovarian syndrome who are at high risk of ovarian hyperstimulation syndrome (Chian *et al.* 2000).

It has been suggested that IVM can be a potential technique in assisted reproductive technology (ART). However, nuclear maturity needs to be synchronised with cytoplasmic maturation, otherwise different morphological abnormalities will be observed in the oocytes (Rienzi *et al.* 2008). Conversely, oocyte quality can be a determining factor in the outcome of ART cycles. One of the major predictors of oocyte quality is the morphology of the oocytes (Khalili *et al.* 2013). Recently, zona pellucida (ZP) birefringence and meiotic spindle visualisation, as determined using the PolScope (OCTAX PolarAID; Octax, Herbon, Germany), have become reliable markers of oocyte quality. The PolScope system is a non-invasive technique to assess birefringent structures such as the meiotic spindle and ZP in living oocytes (Rienzi *et al.* 2004). It could be used to help select suitable oocytes for insemination in intracytoplasmic sperm injection (ICSI; Khalili *et al.* 2012). In addition, it has been confirmed that the PolScope has no negative effects on mouse and human oocytes and embryos (Liu *et al.* 2000*a*).

During the transition from the MI to MII stage, the meiotic spindle is a dynamic structure (Montag *et al.* 2006). Wang *et al.* (2001) reported that the presence of a birefringent spindle in MII oocytes increases the fertilisation rate and embryo

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development. In addition, Li *et al.* (2006) revealed that spindle and chromosome organisation of immature oocytes could be affected by the IVM program. Disturbance of the meiotic spindle in oocytes is due to abnormal chromosome alignment and subsequent aneuploidy in embryos from these oocytes (Wang *et al.* 2001).

The ZP is a multilaminar glycoprotein coat composed of filaments organised in different orientations surrounding the maturing oocytes during ovulation, fertilisation and early embryonic development (Familiari *et al.* 2008). It has been reported that ZP birefringence may predict the quality of MII oocytes (Braga *et al.* 2010), although Petersen *et al.* (2011) reported that ZP birefringence was not a prognostic tool for oocyte selection. The aim of the present study was to evaluate meiotic spindle and ZP birefringence in *in vivo*-matured and IVM oocytes from the same patients to determine whether these structures are influenced by IVM technology. Furthermore, we analysed correlations between the morphology of the oocytes and both ZP birefringence and the presence of the meiotic spindle.

Materials and methods

Participants

In this prospective study, oocytes were collected from 42 patients (mean (± s.d.) age 28.5 ± 4.5 years) undergoing oocyte retrieval for ICSI at the Research and Clinical Center for Infertility (Yazd, Iran). The study population comprised patients who had both mature (MII) and immature (GV and MI) oocytes. The present study was approved by Ethics Committee of our institution Research and Clinical Center for Infertility (Yazd, Iran).

In all, 85 immature oocytes (47 GV, 38 MI) and 85 MII oocytes were obtained from the patients. The same number of MII and immature oocytes was obtained from each patient. At first, the MII oocytes were evaluated for presence of a spindle, ZP birefringence intensity and morphological status; then, they were used for clinical procedures. The immature oocytes were cultured in IVM medium (SAGE IVF, Trumbull, CT, USA) for 24–40 h to allow polar body extrusion. The IVM oocytes were assessed and compared with *in vivo*-matured MII oocytes in terms of spindle and ZP birefringence. Finally, the relationship between morphology and these two parameters was evaluated.

Ovarian stimulation and oocyte preparation

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). When adequate follicular growth was observed on transvaginal ultrasound, recombinant human chorionic gonadotrophin (rhCG; Ovidrel; Serono) was administered to trigger final maturation and ovulation. Thirty-six hours 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% CO_2 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 IU mL^{-1} hyaluronidase (Irvine Scientific, Santa Ana, CA, USA) and by pipetting the COCs with a pasture pipette. The denuded oocytes were classified as either mature (MII) oocytes used for ICSI procedures or immature (GV or MI) oocytes that were cultured *in vitro*.

IVM procedures

Initially, immature oocytes were washed in 3 drops of washing medium (SAGE IVF) before being cultured in maturation medium (SAGE IVF) supplemented with 75 mIU mL^{-1} FSH and 75 mIU mL^{-1} LH (Ferring) at 37°C in an incubator under 5% CO_2 and 95% air with high humidity. The maturity of the oocytes was assessed using a stereomicroscope (Olympus, Tokyo, Japan) between 24 and 40 h.

Imaging of the ZP and meiotic spindle

To evaluate ZP birefringence and spindle imaging, each mature oocyte was placed in a 3-μL droplet of buffered medium (G-Mops-V1; Vitrolife) in a glass-bottomed culture dish (WillCo-Dish; Bellco Glass New Jersey, USA) covered with warm mineral oil (Irvine Scientific). The oocytes were observed under an inverted microscope (TE300; Nikon, Tokyo, Japan) mobilised with a stage heated to 37°C and a polarising optical system (OCTAX PolarAIDE; Octax). This system reveals birefringent structures, such as the ZP and spindle, using OCTAX Eyeware software. The oocytes were screened to evaluate ZP birefringence and visualisation of the meiotic spindle. ZP scoring was automatic and oocytes were classified as having a high or low birefringent ZP.

Assessment of oocyte morphology

The morphology of *in vivo*-matured and IVM MII oocytes was evaluated after denudation of cumulus cells and IVM, respectively, under an inverted microscope (TE300; Nikon). The morphology of oocytes was categorised on the basis of the presence of intra- and extracytoplasmic abnormalities. Intracytoplasmic abnormalities included dark oocytes, the presence of vacuoles, refractile bodies (RF), smooth endoplasmic reticulum clusters (SERc), ooplasm granulation and bull eye (central aggregation of organelles and vesicles within ooplasm). Extracytoplasmic abnormalities included an irregular shape, a dark or wide ZP, a wide perivitelline space (PVS), PVS debris and a fragmented polar body (fPB; Xia 1997; Balaban *et al.* 1998; Mikkelsen and Lindenberg 2001; Balaban and Urman 2006).

Statistical analysis

Data are presented as the mean ± s.d. or as odds ratios (OR) with 95% confidence intervals (CI), as appropriate. Data were compared using Chi-squared and Fisher's exact tests. Data were analysed using SPSS version 18 (SPSS, Chicago, IL, USA). Differences were considered significant at $P < 0.05$.

Results

Outcomes of oocyte IVM

In all, 434 oocytes were retrieved, of which 13 (3%) were discarded after exposure to hyaluronidase because of an abnormal

Table 1. Morphological evaluations *in vivo*-matured and IVM oocytes
OR, odds ratio; CI, confidence interval; SERc, smooth endoplasmic reticulum cluster; ZP, zona pellucida; PVS, perivitelline space; PB, polar body

Variables	*In vivo*-matured oocytes	IVM oocytes	OR (95% CI)	*P*-value
Normal oocytes	21.4%	5.3%		
Intracytoplasmic abnormality				
Irregular shape	3.6%	26.8%	0.10 (0.02–0.46)	0.001
Vacuole	4.3%	16.1%	0.29 (0.07–1.15)	NS
Refractile body	28.6%	62.5%	0.24 (0.10–0.53)	0.001
SERc	7.1%	19.6%	0.31 (0.09–1.05)	NS
Ooplasm granulation	19.6%	14.3%	1.46 (0.54–3.97)	NS
Dark oocyte	8.9%	8.9%	1.00 (0.27–3.66)	NS
Extracytoplasmic abnormality				
Dark ZP	19.6%	19.6%	1.00 (0.39–2.54)	NS
Wide ZP	28.6%	30.4%	0.91 (0.40–2.06)	NS
Wide PVS	33.9%	37.5%	0.85 (0.39–1.85)	NS
PVS debris	14.3%	19.6%	0.68 (0.25–1.84)	NS
Bull eye (central aggregation of organelles and vesicles within ooplasm)	10.7%	16.1%	0.62 (0.20–1.89)	NS
Fragmented PB	26.8%	48.2%	0.39 (0.17–0.86)	0.031

Table 2. Zona pellucida birefringence and spindle visualisation in *in vivo*-matured and IVM oocytes
Unless indicated otherwise, data show the number of oocytes in each group, with percentages given in parentheses. OR, odds ratio; CI, confidence interval; ZP, zona pellucida

	In vivo-matured oocytes	IVM oocytes	OR (95% CI)	*P*-value
ZP birefringence				
High	38 (67.9%)	43 (76.8%)	0.63 (0.27–1.47)	0.39
Low	18 (32.1%)	13 (23.2%)		
Spindle visualisation				
Visible	32 (57.1%)	25 (44.6%)	1.65 (0.78–3.48)	0.25
Not visible	24 (42.9%)	31 (55.4%)		

appearance (e.g. extensive vacuolisation or degeneration). Of the remaining 421 oocytes, 336 (79.8%) were MII, whereas 47 (11.2%) and 38 (9%) were GV and MI oocytes, respectively. After IVM, 28 (59.6%) GV and 28 (73.7%) MI oocytes reached maturity ($P = 0.25$). The maturation rate was lower for IVM (65.9%) compared with *in vivo*-matured oocytes (79.8%; $P = 0.007$). Moreover, in the case of 10 patients, all immature oocytes (11 GV and two MI) showed signs of degeneration or arrest after IVM.

Morphologic analysis of oocytes

In the present study, 12 morphologic parameters were compared between *in vivo*-matured and IVM MII oocytes (Table 1). Subanalysis revealed no significant differences in extracytoplasmic abnormalities between the two groups, with the exception of fPB, which was higher in the IVM group ($P = 0.03$). In terms of intracytoplasmic abnormalities, the occurrence of an irregular shape and RF differed significantly ($P = 0.001$) between the two groups. However, the occurrence of a dark oocyte and a dark ZP was similar between the *in vivo*-matured and IVM oocytes ($P = 1.00$). The data also reveal that the most frequent abnormality in oocyte morphology was a wide PVS and RF in *in vivo*-matured and IVM MII oocytes, respectively. The percentage of morphologically normal oocytes in the *in vivo*-matured and IVM groups was 21.4% and 5.3%, respectively. Oocytes retrieved from one patient (31 years old) were found to have an irregular shape and wide PVS (four immature and four mature oocytes).

Meiotic spindle visualisation and ZP birefringence scores

There was no significant relationship between a high birefringent ZP and either *in vivo*-maturation or IVM of oocytes ($P = 0.39$). However, the percentage of a high birefringent ZP was higher in the IVM than *in vivo*-matured group (76.8% vs 67.9%, respectively; Table 2; Fig. 2). Furthermore, there were no significant differences in the percentage of a high birefringent ZP in oocytes derived from GV or MI oocytes (51.2% vs 48.8%, respectively; $P = 1.00$). A birefringent spindle was

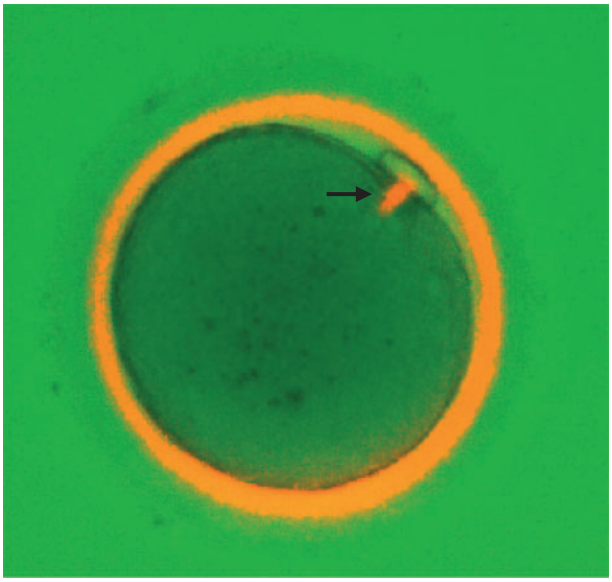

Fig. 1. PolScope (OCTAX PolarAID; Octax, Herbon, Germany) image of an IVM human oocyte. A bright meiotic spindle (arrow) is clearly visible.

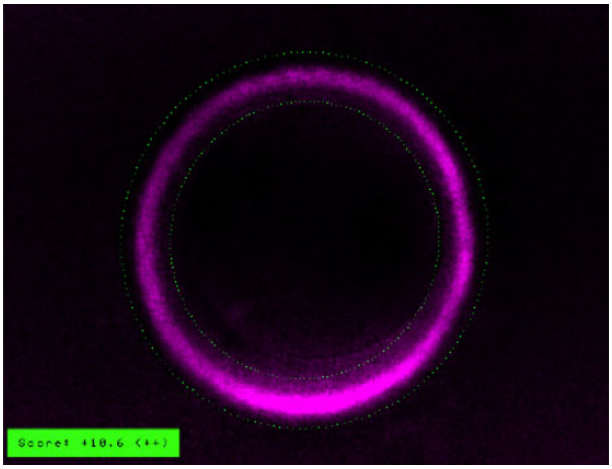

Fig. 2. Inner layer of the zona pellucida imaged using the PolScope (OCTAX PolarAID; Octax, Herbon, Germany).

detected in 57.1% of *in vivo*-matured MII oocytes, compared with 44.6% of IVM MII oocytes ($P = 0.25$; Table 2; Fig. 1). The rate of meiotic spindle visualisation after IVM was similar in oocytes derived from GV and MI oocytes (60% vs 40%, respectively; $P = 0.28$). There was no correlation between oocyte ZP score and spindle visualisation in either group.

Correlation between oocyte morphology and ZP birefringence and spindle visualisation

Oocytes were divided into four groups on the basis of the type of abnormality detected, as follows: (1) those with intracytoplasmic abnormalities; (2) those with extracytoplasmic abnormalities; (3) those with both types of abnormalities; and (4) normal oocytes. As indicated in Table 3, *in vivo*-matured oocytes with intracytoplasmic and both types of abnormalities had a significantly higher percentage of low birefringent ZP ($P = 0.007$ (OR 0.12; 95% CI 0.02–0.62); $P = 0.02$ (OR 0.2; 95% CI 0.05–0.75), respectively). The normal IVM oocytes had a high birefringent ZP. Nevertheless, there was no significant correlation between morphological abnormalities and ZP birefringence in IVM and *in vivo*-matured MII oocytes ($P < 0.05$). Furthermore, there was no significant relationship between morphology and spindle detection in either the *in vivo*-matured or IVM MII oocytes. However, there was a tendency for a higher percentage of abnormal oocytes to have an invisible spindle, although the difference did not reach statistical significance (Table 4).

Discussion

The oocyte is a complex cell with organelles and structures such as a meiotic spindle, cortical granules, mitochondria and ZP, each of which needs to be in an appropriate condition for cell maturation (Trimarchi and Keefe 2006). Many studies have reported that oocyte morphology has no relationship with ART outcomes (Sutter *et al.* 1996; Balaban *et al.* 1998). However, Khalili *et al.* (2005) and Xia (1997) demonstrated that normal oocyte morphology has the most important role in generating high-grade embryos. In addition, it has been reported that high-quality embryos with rapid cleavage rates are obtained following IVM if normal oocytes rather than those with abnormalities are used (Mikkelsen and Lindenberg 2001). Because IVM has become a potential ART technique, the status of oocytes after

Table 3. Correlation between morphology and zona pellucida birefringence in *in vivo*-matured and IVM oocytes
OR, odds ratio; CI, confidence interval; ZP, zona pellucida; ICA, intracytoplasmic abnormality; ECA, extracytoplasmic abnormality

	In vivo-matured oocytes				IVM oocytes			
	ZP birefringence		OR (95% CI)	*P*-value	ZP birefringence		OR (95% CI)	*P*-value
	High	Low			High	Low		
ICA	50%	88.9%	0.12 (0.02–0.62)	0.007	83.7%	100%	0.73 (0.62–0.86)	NS
ECA	63.2%	77.8%	0.49 (0.13–1.78)	NS	81.4%	92.3%	0.36 (0.04–3.22)	NS
Both ICA and ECA	42.1%	77.8%	0.20 (0.05–0.75)	0.021	72.1%	92.3%	0.21 (0.02–1.84)	NS
No abnormalities	26.33%	11.1%	2.85 (0.55–14.6)	NS	9.3%	0%	1.33 (1.14–1.55)	NS

Table 4. Correlation between morphology and spindle visualisation in *in vivo*-matured and IVM oocytes
OR, odds ratio; CI, confidence interval; ZP, zona pellucida; ICA, intracytoplasmic abnormality; ECA, extracytoplasmic abnormality

	In vivo-matured oocytes				IVM oocytes			
	Spindle detected		OR (95% CI)	*P*-value	Spindle detected		OR (95% CI)	*P*-value
	Yes	No			Yes	No		
ICA	53.1	75	0.37 (0.11–1.20)	NS	84	90.3	0.56 (0.11–2.78)	NS
ECA	59.4	79.2	0.38 (0.11–1.29)	NS	84	83.9	1.01 (0.24–4.24)	NS
Both ICA and ECA	43.8	66.7	0.38 (0.13–1.16)	NS	76	77.4	0.92 (0.26–3.20)	NS
No abnormalities	28.1	12.5	2.73 (0.65–11.4)	NS	8	6.5	1.26 (0.16–9.64)	NS

IVM was assessed in the present study and compared with oocytes matured *in vivo*.

We found that 20% of retrieved oocytes remained immature, which is slightly higher than in other reports (Van Steirteghem *et al.* 1993). However, despite ovarian hyperstimulation, some oocytes remain immature, and this may be due to intrinsic defects in the oocytes or even the follicles (Halvaei *et al.* 2012). Almost all oocyte dysmorphisms were higher following IVM compared with *in vivo* maturation to the MII stage. There were no significant differences in the rate of oocyte abnormalities between the two groups, with the exception of irregular shape, RF fPB. The most common dysmorphism in the *in vivo*-matured group was the extracytoplasmic abnormality of a wide PVS, whereas in the IVM group the most common anomaly was intracytoplasmic (RF). Recently, Halvaei *et al.* (2012) and Rienzi *et al.* (2008) reported that the most common anomaly in *in vivo*-matured oocytes was fPB. Conversely, Nazari *et al.* (2011) evaluated the morphology of IVM oocytes and found that RF was the highest abnormality. The wide PVS detected in the present study is most likely the outcome of both a decrease in oocyte diameter and an increase in the inner diameter of the ZP. In addition, a wide PVS and fPB may be related to post matured oocyte (aged oocyte) because of oocyte aging (Miao *et al.* 2004). Balaban and Urman (2006) reported that extracytoplasmic anomalies (e.g. a wide PVS) were only a phenotypic heterogeneity of the oocytes. Recently, ultrastructural assessment revealed that after IVM the major abnormality was related to numerous large mitochondria-vesicle (MV) complexes in oocytes compared with *in vivo*-matured oocytes (Shahedi *et al.* 2013). Another recent study concluded that human oocytes subjected to IVM exhibit good quality and fine structural morphology because of the absence of cytoplasmic vacuolisation (Khalili *et al.* 2013).

Recently, assessment of the meiotic spindle and ZP birefringent intensity in live human oocytes using polarised light microscopy has been suggested as a marker of oocyte quality (Raju *et al.* 2007). The ZP is composed of three layers that cause directional variations in its filaments. Therefore, these layers exhibit different birefringent with application of the PolScope. The inner layer exhibits maximum birefringent and is primarily accountable for changes in the birefringence of the entire ZP (Pelletier *et al.* 2004). The meiotic spindle, a bipolar structure composed of microtubules, controls chromosome movement and alignment through different stages of meiosis (Raju *et al.* 2007). Liu *et al.* (2000*b*) showed that oocytes in which a spindle could not be visualised using the PolScope had a disassembled spindle when visualised by confocal microscopy. During IVM, oocytes may be exposed to chemicals and physical changes that affect the spindle microtubules, which could result in a decrease in the developmental potential of embryos (Sun *et al.* 2004; Li *et al.* 2006). We hypothesised that the IVM protocol may have some deleterious effects on ZP birefringence and meiotic spindle visualisation. However, we did not notice any significant differences in these parameters between the *in vivo*-matured and IVM groups. However, the percentage of oocytes in which a spindle was detected was higher in the *in vivo*-matured compared with the IVM group. These findings are similar to those reported by Fang *et al.* (2007). However, in that study, Fang *et al.* (2007) selected oocytes from two different groups of patients; in the present study all the oocytes were from a single patient population. The data generated from the present study confirm the results of Li *et al.* (2006), who evaluated the oocyte meiotic spindle by immunocytological staining and confocal microscopy and found that there was a higher proportion of abnormal spindles in the IVM compared with *in vivo*-matured oocytes. In addition, it has been reported that there is a lower rate of spindle presentation in oocytes after IVM than in oocytes matured *in vivo* (Rienzi *et al.* 2005). Conversely, one study observed that a visible meiotic spindle was detected in most IVM oocytes (Braga *et al.* 2008). Rienzi *et al.* (2004) detected the spindle in 100% of *in vivo*-matured MII oocytes. These differences among studies may be due to technical advances because Rienzi *et al.* (2004) rotated each oocyte with an injection pipette while making observations.

In the present study, the highest percentage of high ZP birefringence was found in the IVM oocytes. Some authors have reported that ZP birefringence is unaffected during IVM (Braga *et al.* 2010; Petersen *et al.* 2011). In addition, these authors demonstrated no correlation between ZP birefringence scores and *in vitro* nuclear and cytoplasmic maturity . The features of the ZP layers may reflect oocyte cytoplasmic maturation, whereas different maturity stages and environmental factors (e.g. culture conditions) can affect ZP construction (Liu *et al.* 2003; Braga *et al.* 2010). According to Van Blerkom (1996), imperfect cytoplasmic maturation or asynchrony between nuclear and cytoplasmic maturation may be due to meiotic defects. In all abnormal oocytes detected in the present study, the rate of low ZP birefringence tended to be higher than

that of high ZP birefringence, but the differences failed to reach statistical significance expect in the case of *in vivo*-matured MII oocytes with only intracytoplasmic anomaly and those with both intra- and extracytoplasmic anomalies.

In conclusion, ZP birefringence, and meiotic spindle visualisation and normal architecture of human oocytes are not negatively affected by IVM. Thus, IVM can be considered a safe technology for the maturation and maintenance of integrity of human oocytes.

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