Meiotic spindle and zona pellucida characteristics as predictors of embryonic development: a preliminary study using polscope imaging

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Abstract

This study assesses meiotic spindle and zona pellucida characteristics using the polscope, and analyses their relationship to embryonic development potential. A total of 205 matured oocytes retrieved from 25 patients undergoing ovarian stimulation were imaged for meiotic spindle and zona pellucida characteristics using the polscope. After intracytoplasmic sperm injection, the oocytes were cultured and assessed for progression to blastocysts. Meiotic spindles were visualized in 78.0% of oocytes. Significantly more oocytes with visible spindles fertilized and progressed to blastocysts compared with oocytes without visible spindles. Oocytes with spindle retardance of >3 nm showed a greater progression to blastocysts compared with those with a retardance of 2-3 nm or <2 nm. More blastocysts were obtained from oocytes with spindle lengths of >12 nm than from oocytes with spindle lengths 10-12 nm or <10 nm. A difference in progression to blastocyst was observed in oocytes with a zona inner layer retardance of >3 nm compared with oocytes with retardance of 2-3 nm or <2 nm. Oocytes with an inner layer zona of 10–12 nm thickness showed better progression compared with those with a thickness of 8–10 nm or <8 nm. Quantitative measurement of length and retardance of the meiotic spindle and zona pellucida has a positive predictive value in relation to embryonic development.

Keywords: *embryonic development, human oocyte, meiotic spindle, polscope, retardance*

Introduction

Rapid strides in development of various aspects of assisted reproduction have provided many insights into human embryonic development. With the ultimate aim of achieving a pregnancy following single embryo transfer, various workers have attempted to identify oocyte parameters which can predict a favourable outcome (Serhal *et al.*, 1997; Xia, 1997; Borini *et al.*, 2005). With conventional light microscope imaging, oocyte characteristics such as cumulus, polar body, cytoplasm and zona pellucida morphology have been related to embryonic development and pregnancy rates. However, no single characteristic has been infallible in predicting the ability of oocytes to achieve a pregnancy. Hence, efforts to identify reliable parameters for the assessment of oocyte quality remain a priority.

One such parameter that plays a major role in meiosis and development is the meiotic spindle. Until 1990, most of the work on spindles was performed with the fluorescent microscope, which provided extensive information on the meiotic spindle and its role in cell division. However, its routine use in assisted reproduction procedures is precluded by its inability to be applied to live oocytes. With the advent of the polscope, it has become possible to assess meiotic spindle and zona pellucida characteristics in live oocytes. Moreover, this procedure has the advantage of being totally non-invasive and preserves the oocyte viability. The aim of the present investigation was to evaluate and analyse meiotic spindle and zona pellucida characteristics of oocytes using the polscope and to assess their influence on embryonic development.

Materials and methods

Patients

The study group consisted of 205 metaphase II (MII) oocytes obtained from 25 patients. The patients' ages ranged from 25 to 40 years, with an average of 30.76 ± 6.2. Only female infertility was included; couples with male infertility were excluded from this study protocol. Indications for assisted reproduction include polycystic ovary disease (n = 10), tubal factor (n = 6), endometriosis (n = 4) and idiopathic (n = 5). The study protocol and the need to perform intracytoplasmic sperm injection (ICSI) and polscope imaging analysis in

spite of the absence of male factor was explained to the couples and they were recruited into the study after their approval. This study was approved by the Institutional Review Board (IRB), Krishna IVF Clinic.

Stimulation protocol and oocyte collection

Ovarian stimulation was achieved using gonadotrophin-releasing hormone (GnRH) agonist and recombinant FSH. Down-regulation was initiated using intramuscular injection of triptorelin (Decapeptyl; Ferring Pharmaceuticals, Mumbai, India) 3.75 mg on day 21 of the cycle. Adequacy of down-regulation was confirmed by measuring oestradiol (<50 pg/ml) and LH concentrations (<1 ng/ml). Ovarian stimulation was achieved using recombinant FSH (Recagon; Organon, USA) and the dose was adjusted on the basis of individual response. Human chorionic gonadotrophin (HCG) at a dose of 10,000 IU was given after two follicles of 18 mm or more were visualized in the ultrasound scan. Oocyte retrieval was scheduled 36 h later by transvaginal ultrasonography (TVS) guided aspiration. Cumulus cells were removed using hyaluronidase (Sigma, USA) and maturation of the oocytes was confirmed by the presence of the first polar body. The oocytes were washed twice in Gamete solution (Vitrolife, Sweden) and placed in an incubator for 3–4 h before spindle imaging.

Visualization of spindle using the polscope

For spindle imaging, each oocyte was placed in an equilibrated 5 μl media droplet overlaid with mineral oil in a glass bottomed culture dish (Willco Wells, Netherlands). Oocytes were imaged using a Zeiss Axiovert 200M microscope equipped with an LC polscope (CRI, USA) controller and CCD camera. The images were acquired at ×200 magnification and the location, length, mean retardance of the meiotic spindle and the mean retardance of inner layer of zona pellucida and its thickness were analysed using an LC computerized image analysis system. A temperature of 37°C was maintained throughout the procedure using an incubator (Incubator XL; Carl Zeiss, Germany) fitted onto the microscope. In order to optimize the spindle and polar body visualization, oocytes were rotated using the holding pipette. ICSI was carried out by using one of the following methods. For oocytes with birefringent spindle, sperm injection was performed after placing the spindle at the 6 o'clock position. For oocytes with shift of meiotic spindle from the polar body, sperm injection was performed aligning the imaged spindle to the 6 o'clock position. For oocytes without spindles, sperm injection was performed after placing the polar body at the 6 o'clock position. After ICSI, oocytes with and without spindles were washed twice and cultured separately and sequentially in fertilization media droplets (IVF; Vitrolife) for further analysis.

Assessment of fertilization

Oocytes were examined under a stereomicroscope for fertilization 16–18 h after ICSI. Oocytes with two pronuclei and having a second polar body were considered as normally fertilized. The fertilized oocytes were washed twice and cultured in fresh droplets of cleavage media (G1; Vitrolife). After 3 days post-ICSI, embryos were transferred to blastocyst media (G2; Vitrolife).

Statistical analysis

The results were compared using the chi-squared test and one-way analysis of variance (ANOVA). The values were expressed as ± SEM. A *P*-value of <0.05 was considered statistically significant.

Results

A total of 205 oocytes from 25 patients were examined with a polscope. The meiotic spindle was visualized in 160 oocytes (78.0%), but could not be visualized in the remaining 45 oocytes (22.0%). Significantly (*P* <0.05) more oocytes with visible spindles fertilized (82.5 versus 31.1%) and progressed to the blastocyst stage on day 5 (48.4 versus 14.2%) compared with the oocytes without visible spindles (**Table 1**).

Table 1. Influence of the presence of polscope-visualised meiotic spindle on embryonic development.

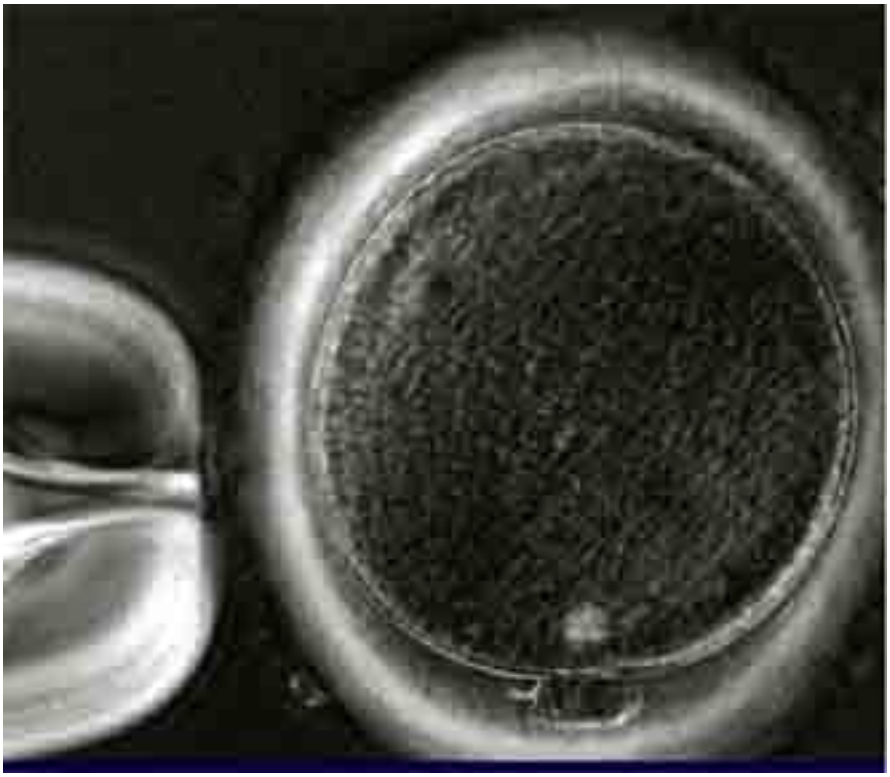
Polscope visualisation	*No. of oocytes (%)*	*No. fertilized oocytes (%)*	*No. proceeding to blastocyst (%)*

With spindle	160 (78.0)	132[a] (82.5)	64[b] (48.5)
Without spindle	45 (22.0)	14[a] (31.1)	2[b] (14.3)

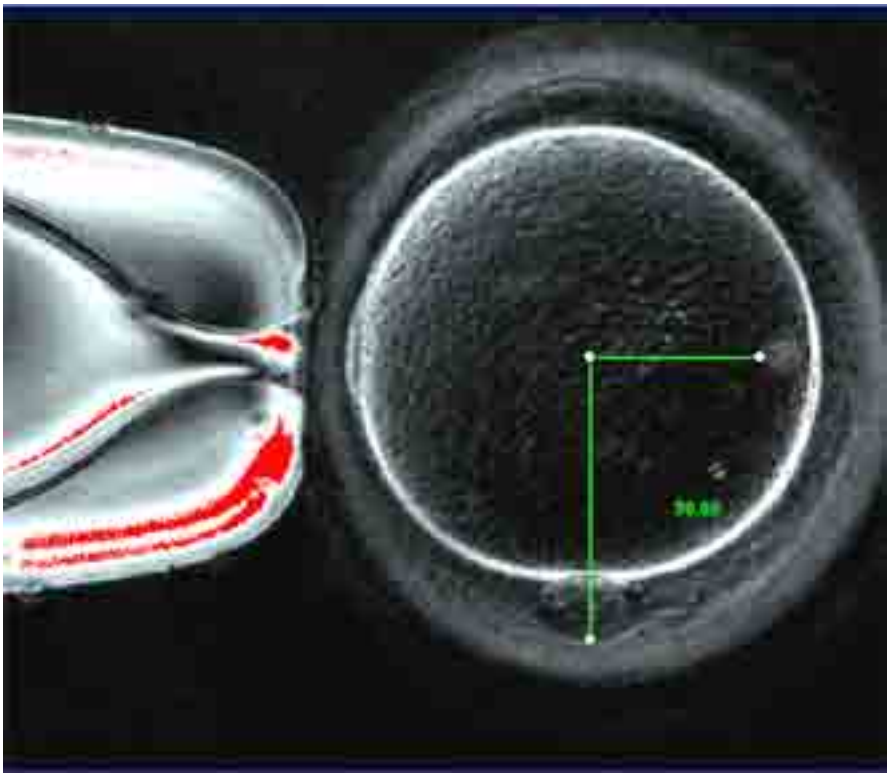
[a,b]Values with the same superscript letter are significantly different ($P < 0.05$; chi-squared test).

Angle of spindle relative to polar body versus embryonic development

Mature (MII) oocytes had spindles at various angles relative to the first polar body (**Figure 1a**,**b**). These oocytes were classified into four groups on the basis of the shift from the polar body. Oocytes in group A showed a 0–5° shift, in group B showed a 6–45° shift, in group C showed a 46–90° shift and those in group D showed a >90° shift. Among the 160 oocytes with visible spindle, 30.0% were categorized into group A, 38.8% into group B, 18.1% into group C and 13.1% into group D (**Table 2**). After ICSI, no significant differences in the blastocyst progression rates were observed among the different groups.



(**a**)



(**b**)

Figure 1. Oocytes with meiotic spindle located (**a**) adjacent to polar body and (**b**) with 90° shift from polar body. Magnification ×200.

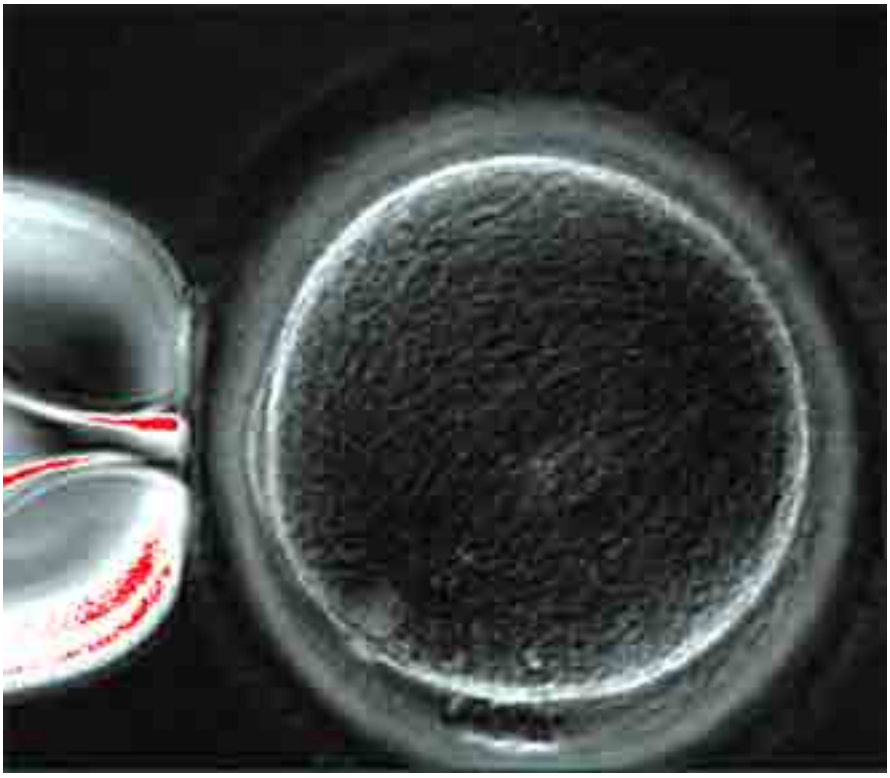
Table 2. Influence of meiotic spindle shift relative to polar body on embryonic development.

Group	*Spindle angle to polar body*	*No. of oocytes*	*No. proceeding to blastocysts (%)*
A	0–5°	48	20 (41.7)
B	6–45°	62	23 (37.1)
C	46–90°	29	12 (41.4)
D	>90°	21	9 (42.9)

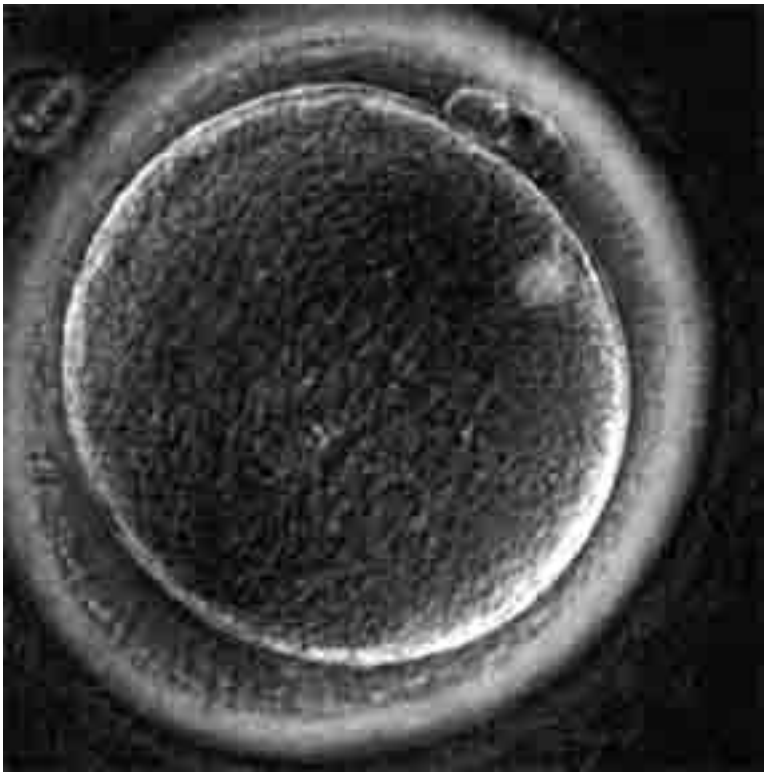
There were no statistically significant differences in blastocyst progression rate between the groups.

Spindle retardance versus embryonic development

The development of an embryo to a blastocyst was taken as the criterion for good embryonic development (**Figure 2a**,**b**). Among 160 oocytes with visible spindle, 40% progressed to blastocysts. Of these, 60.9% showed retardance of >3 nm, 25.0% showed retardance between 2 and 3 nm and the remaining 14.1% showed retardance of <2 nm (**Table 3**). Significantly more oocytes with spindle retardance of >3 nm progressed to blastocysts when compared with oocytes having retardance between 2–3 and <2 nm respectively ($P < 0.05$).



(**a**)



(**b**)

Figure 2. Polscope imaging of meiotic spindle. Oocytes exhibiting (**a**) weak retardance and (**b**) normal retardance. Magnification ×200.

Table 3. Influence of mean meiotic mean spindle retardance on embryonic development.

Spindle retardance (nm)	*No of blastocysts (%)*
>3.0	39 (60.9)[a,b]
2–3	16 (25.0)[a]
<2.0	9 (14.1)[b]

[a,b]Values with the same superscript letter are significantly different ($P < 0.05$; chi-squared test).

Spindle length versus embryonic development

The length of the spindle in its long axis was measured using the polscope software and the results were analysed with respect to blastocyst progression. Among the 64 oocytes which progressed to blastocysts, 45.3% had a spindle length between 12–15 nm, 34.4% had a spindle length between 10–12 nm and the remaining 20.3% had a spindle length of <10 nm (**Table 4**). Oocytes with spindle lengths between 12 and 15 nm progressed to a significantly higher number of blastocysts ($P < 0.05$) when compared with oocytes having spindle lengths between 10–12 nm and <10 nm respectively.

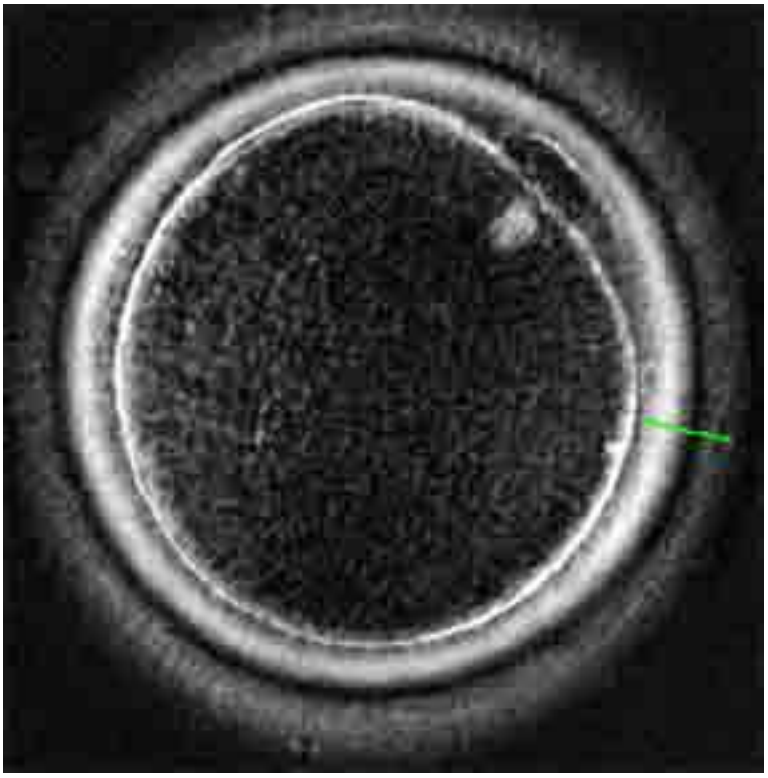
Table 4. Influence of meiotic spindle length on embryonic development.

Spindle length (nm)	*No. of blastocysts (%)*
12–15	29[a,b] (45.3)
10–12	22[a] (34.4)
<10	13[b] (20.3)

[a,b]Values with the same superscript letter are significantly different ($P < 0.05$; chi-squared test).

Inner zona retardance versus embryonic development

In the present study, the retardance of the inner layer of the zona was measured prior to ICSI. Among 64 oocytes which progressed to blastocysts, 56.3% had a zona retardance of >3 nm, 26.6% showed retardance between 2 and 3 nm and the remaining 17.2% showed a retardance of <2 nm. Significantly ($P < 0.05$) more oocytes having zona retardance of >3 nm progressed to blastocysts on day 5 when compared with oocytes having a zona retardance between 2 and 3 nm and <2 nm respectively (**Table 5** and **Figure 3a**,**b**).



(**a**)

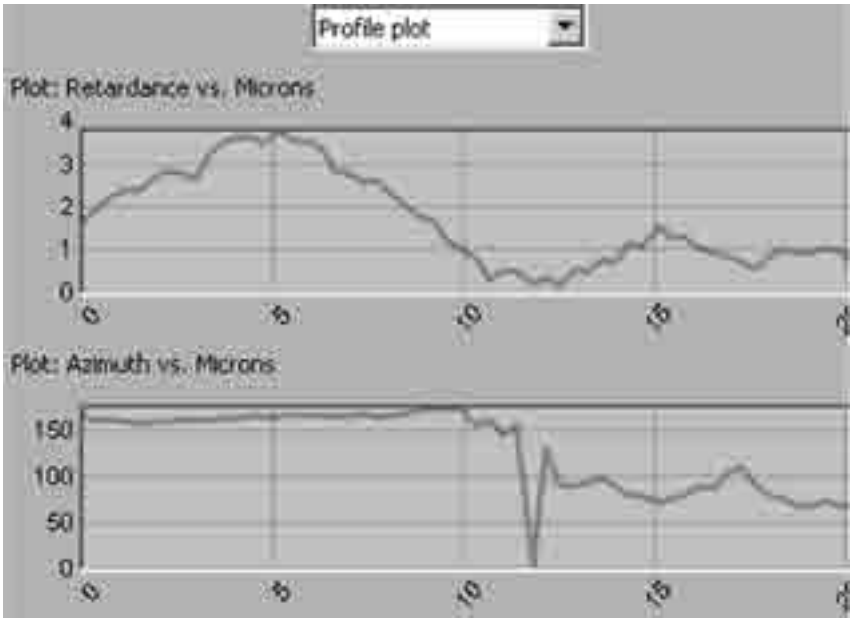


Figure 3. (**a**) An ideal oocyte displaying good spindle retardance and length, trilaminar structure of zona with good retardance of inner layer. Magnification ×200. (**b**) Retardance profile of the three layers of zona pellucida.

Table 5. Influence of mean retardance of the inner layer of zona pellucida on embryonic development.

Retardance of inner layer of zona (nm)	*No of blastocysts (%)*
>3.0	36[a,b] (56.3)
2–3	17[a] (26.6)
<2.0	11[b] (17.2)

[a,b]Values with the same superscript letter are significantly different ($P < 0.05$; chi-squared test).

Inner zona thickness versus embryonic development

In human oocytes, the innermost layer (IL) of zona pellucida is the thickest among the three zona layers. In the present study, thickness of the IL of zona pellucida was measured before ICSI. Among 64 oocytes that progressed to blastocysts, 53.1% had an IL zona thickness of 10–12 nm, 25.0% showed a thickness between 8 and 10 nm and the remaining 21.9% showed a thickness of <8 nm. Oocytes with IL zona thickness between 10 and 12 nm progressed significantly more frequently ($P < 0.05$) to blastocysts compared with oocytes having IL zona thicknesses between 8–10 nm or <8 nm (**Table 6**).

Table 6. Influence of thickness of the inner layer of zona pellucida on embryonic development.

Thickness of inner layer of zona (nm)	*No. of blastocysts (%)*
10–12	34[a,b] (53.1)
8–10	16[a] (25.0)
<8	14[b] (21.9)

[a,b]Values with the same superscript letter are significantly different ($P < 0.05$; chi-squared test).

Spindle location in various age groups

After analysing the oocytes of patients in different age groups, no significant relationship was observed between the degree of spindle shift and age of the patient (**Table 7**).

Table 7. Meiotic spindle location in various age groups.

Spindle angle to polar body	*Percent of oocytes*		
	25–29 years old[a]	*30–35 years old*[b]	*>35 years old*[c]
0–5°	36.0	37.5	35.2
6–45°	44.3	45.1	46.4
46–90°	13.7	10.9	12.9
>90°	6.0	6.5	5.5

[a]108 oocytes; [b]35 oocytes; [c]17oocytes. There were no statistically significant differences between the age groups.

Maternal age versus spindle retardance and length

A significant change in retardance and also the length of spindle with age of patients was observed (**Table 8**, $P < 0.05$). With increase in age there was a decrease in retardance and length of the spindle.

Table 8. Influence of maternal age on meiotic spindle retardance and length.

	Age (years)		
	25–29[a]	*30–35*[b]	*>35*[c]
Retardance (nm)	3.01 ± 0.14[d,e]	2.22 ± 0.07[d]	1.84 ± 0.06[e]
Spindle length (nm)	14.87 ± 0.36[f,g]	12.42 ± 0.30[f]	10.52 ± 0.58[g]

[a]108 oocytes; [b]35 oocytes; [c]17oocytes; [d-g]Values with the same superscript letter are significantly different ($P < 0.05$; chi-squared test).

Discussion

The identification of reliable criteria for assessment of oocyte development potential prior to fertilization is one of the most investigated areas in the field of assisted reproduction. Attempts to establish reliable parameters indicative of oocyte development potential have involved assessment of cumulus and zona morphology, cytoplasmic features such as granulation, vacuoles and refractile bodies, and polar body features such as size and fragmentation. Conflicting reports have been published by various authors for the above parameters in relation to embryonic development (Desutter *et al.*, 1996; Serhal *et al.*, 1997; Balaban *et al.*, 1998, 2001; Loutradis *et al.,* 1999; Rattanachaiyanon *et al.,* 1999; Verlinsky *et al.*, 2003; Ebner *et al.*, 2006).

For the past decade, the meiotic spindle has been extensively investigated as a possible predictive feature for oocyte selection. Most of the earlier studies on meiotic spindle were performed using fluorescent microscopy (Liu *et al.*, 1998). Although this technique gives detailed information about chromosomes and meiotic spindles, its clinical use is limited by its invasive nature and inability to be used for studying live oocytes. Imaging of meiotic spindle in live oocytes has been made possible by the development of an orientation-independent polarized light microscope, the polscope, which facilitates study of the spindle's architecture non-invasively in live human oocytes without affecting viability (Oldenbourg, 1996; Liu *et al.*, 2000a; Keefe *et al.*, 2003). Unlike conventional methods of imaging spindles, the polscope does not require preparative techniques such as fixation and staining and has a unique potential to visualize and measure birefringent structures dynamically and non-invasively in living cells. The polscope operates on the principle that when two orthogonally polarized light rays pass through orderly arranged filamentous structures such as spindles composed of microtubules, they are differentially slowed down. The resultant change is measured as retardance, and can be quantified (Sato *et al.*, 1975; Oldenbourg, 1999). The measured retardance is directly proportional to the density of the microtubules. This relationship between spindle retardance and microtubule density was first established by Sato *et al.* (1975). Liu *et al.* (2000b) observed that oocytes with absence of spindle when visualized with a polscope exhibited disassembled spindles and misalignment of chromosomes when visualized by confocal microscopy. Hence, the spindle retardance may be an important parameter for the evaluation of oocyte quality prior to fertilization and further embryonic development (Wang *et al.,* 2001a; Trimarchi *et al.,* 2004).

The meiotic spindle, composed of cytoskeletal microtubules, plays a major role in the successful completion of meiosis by controlling chromosomal movement throughout the various stages of meiosis. Several authors have suggested that disorganization of the spindle leads to disruption of orderly chromosomal segregation, which results in aneuploidy of zygotes (Pickering *et al.*, 1988; Eichenlamb Ritter *et al.*, 2002). The meiotic spindle morphology and kinetics are influenced by several variables such as maternal age, temperature and in-vitro manipulations of oocytes.

Studies by Battaglia *et al.* (1996) indicated that incidence of spindle abnormality in oocytes was significantly higher in older patients. This is further supported by the work of Volarcik *et al.* (1999), who studied age-related effects on the meiotic process of in-vitro matured human oocytes from unstimulated ovaries. This also corresponds with the higher incidence of meiotic segregation errors observed with increased maternal age.

Temperature is an important parameter affecting the integrity of the spindle during handling and culturing of oocytes in in-vitro manipulation. Meiotic spindles in human oocytes are more sensitive to temperature fluctuations when compared with other animals (Pickering *et al.*, 1990; Aman, 1994; Almedia *et al.*, 1995). Disassembled spindles may result from prolonged exposure to low temperature. This is further confirmed by Wang *et al.* (2001b, 2002). A recent study by Sun *et al.* (2004) on in-vitro matured oocytes reported that spindles in human oocytes are also sensitive to temperatures higher than 37°C. Therefore, these results indicate that when human oocytes are manipulated *in vitro*, a rigorous control of 37°C temperature is essential for maintenance of spindle integrity and further embryonic development.

A further potential for injury to the meiotic spindle during in-vitro manipulation of oocytes is the sperm injection procedure. During ICSI, the location of the spindle is assumed to be in relation to the polar body. In order to avoid damage to the spindle, the polar body is positioned at a 90° angle relative to the injection

needle. Studies by Silva *et al.* (1999) and Wang *et al.* (2001c) suggested that the spindle is not always adjacent to the polar body. In such circumstances, where the displaced spindle is in the path of the injecting needle during ICSI, it may be subject to mechanical damage.

Developmental potential of oocytes with visualized spindle using the polscope

Wang and Keefe (2002) reported that oocytes without a visible spindle lead to a high degree of aneuploidy and poor embryonic development. Several authors have suggested that the absence of a spindle may be primarily because of a compromised oocyte with disorganized spindle or secondary to improper control of environmental factors such as temperature control during oocyte retrieval, and micromanipulation procedures (Wang *et al.*, 2001b; Cook *et al.*, 2002). In the present study, it was found that the spindle was absent in 22.0% of mature (MII) oocytes. These findings are comparable with previous studies by Wang *et al.* (2001a), Moon *et al.* (2003) and Cohen *et al.* (2004), whose comparable figures were 18, 16.5 and 24.5% respectively. Furthermore, the fertilization rate of 82.5% in oocytes with visualized spindles was statistically significantly greater compared with that in oocytes with non-visualized spindles (31.1%, P <0.05) (**Table 1**). These findings are in agreement with a previous study by Wang *et al.* (2001a). A significant association between the presence of a spindle and blastocyst progression was also observed (**Table 1**). These results are in accordance with Wang *et al.* (2001c) and Moon *et al.* (2003). However, these results are contrary to those of Cohen *et al.* (2004), who reported no significant difference in embryonic development to day 3 embryos between oocytes with and without spindles.

Role of shift of the spindle from the polar body in embryonic development

Studies on hamster and rhesus monkey oocytes by Silva *et al.* (1999) and Hewitson *et al.* (1999) suggested that the meiotic spindle is not always located adjacent to the polar body. This is further supported by Wang *et al.* (2001c) for live human oocytes. A possible explanation for the shift of the polar body may be due to physical displacement during denudation of cumulus and corona prior to spindle imaging. This explanation is in agreement with previous studies by Rienzi *et al.* (2003, 2005) and Moon *et al.* (2003). In the present study, out of 160 oocytes with visible spindle, 30% had a spindle adjacent to the polar body and the remaining 70% had spindles at different locations, which is in agreement with the above authors. No statistically significant difference was observed between the aligned and non-aligned oocytes in terms of embryonic development. This may be due to the correction of the shift of the spindle during ICSI by aligning the spindle of the oocyte at the 6 o'clock position instead of the polar body.

Spindle retardance and its role in embryonic development

Studies by Sato *et al.* suggested that spindle birefringence was contributed to by microtubules, and also proposed a relationship between spindle retardance and microtubule density (Sato *et al.*, 1975). Trimarchi *et al.* (2004) reported a positive correlation between spindle retardance and embryonic development. A recent study by Shen *et al.* (2006) reported that mean retardance of the spindle is positively related to pronuclear score and better conception rate. In the present investigation, oocytes were categorized on the basis of mean retardance into three groups (**Table 3**). Oocytes with mean retardance of >3.0 progressed to significantly more blastocysts in comparison with other groups (P < 0.05). These findings are in agreement with previous studies by Trimarchi *et al.* (2004) and Shen *et al.* (2006a,b). A trend was also observed between the low retardance, age and oocyte quality in terms of further embryonic development. Studies by De Santis *et al.* (2005) also observed the same trend.

Meiotic spindle length as a predictor of embryonic development

Variation in the meiotic spindle length of oocytes was reported by Wang *et al.* (2001b). The proposed contributing factors that influence the length of spindle are meiotic age (Eichenlaub-Ritter *et al.*, 1986, 1988, 1995) and exposure to different culture conditions. Shen *et al.* (2006) recently reported that the mean spindle lengths of all oocytes in different cohorts are similar. However, in the present study, oocytes with a spindle length of >12 nm showed significantly higher blastocyst progression (**Table 4**; P < 0.05). A decrease in spindle length was also observed with increased maternal age.

Zona inner-layer retardance, a predictive marker for embryonic development

Polscope study reveals the zona pellucida as a three-layer structure, and arrangements of filaments within these layers are different from each other (Oldenbourg, 1997). Due to the different orientation of these filaments, these layers exhibit different birefringence with the polscope. The filaments in the inner layer (IL) of zona are arranged radially and exhibit maximum birefringence, whereas filaments of the outer layer zona (OL) are oriented tangentially and retard the light to a lesser degree, yielding moderate birefringence (Oldenbourg, 1997). The inner and outer layers are separated by the middle layer (ML), which exhibits least birefringence due to random orientation of filaments (Silva *et al.*, 1997). For the past few years, many authors have focused to establish a relationship between these birefringent characteristics of zona pellucida of oocyte and embryonic development (Pelletier *et al.*, 2004; Shen *et al.*, 2005). Studies by Pelletier *et al.* (2004) suggested that the retardance magnitude of zona changes with different maturational stages of the oocyte and embryo. Shen *et al.* (2005) reported a variation in the mean retardance of the IL zona of oocytes in conception and non-conception cycles with a higher mean retardance of the IL zona in the oocytes of conception cycles compared with non conception cycles. They also concluded that the mean retardance of middle and outer layers of zona were not statistically significant. Hence, the retardance magnitude of the inner layer of the zona pellucida has been proposed to have a positive predictive value as a marker for the selection of oocytes with better developmental potential. In the present study oocytes with zona inner layer retardance of >3 nm progressed to significantly more blastocysts in comparison with oocytes with zona inner layer retardance of <3 nm. These findings are in accordance with Shen *et al.* (2005), and further strengthen the importance of zona IL retardance as a predictor of good embryonic development.

Zona thickness versus embryonic development

The thickness of the zona pellucida increases with maturation of the oocyte and becomes thinner in the post-fertilization development (Betrand *et al.*, 1996; Dirnfeld *et al.*, 2003). Shen *et al.* (2005) reported a significant increase in thickness of the inner layer of the zona pellucida in oocytes of conception cycles compared with a non-conception group of oocytes, whereas the thickness of the middle and outer layers of the zona was not significant. In the present study, zona IL thickness was measured and the present findings are in accordance with Shen *et al.*, with oocytes having zona IL thickness between 10 and 12 nm exhibiting the highest blastocyst progression. Further larger prospective studies are needed to validate the role of zona characteristics as a possible positive predictor of good oocyte quality.

In conclusion, the present study shows that the presence of a visible meiotic spindle viewed with a polscope, and quantitative assessment of its mean retardance and length, have a positive predictive value in relation to progression to blastocysts. The measurement of the thickness of the inner layer of the zona pellucida and its mean retardance also shows a similar predictive value. The polscope, by facilitating the analysis of the meiotic spindle and zona pellucida characteristics non-invasively and rapidly, has proven to be a revolutionary tool for assessment of the meiotic spindle. However, further prospective studies with larger patient populations are needed so that the results can be analysed with the patient as the statistical unit rather than the oocyte. Such studies would allow further assessment of the relationship between characteristics of the meiotic spindle and inner layer of zona pellucida and embryonic development, and the various factors which may influence these characteristics.

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