Article

Are there non-invasive markers in human oocytes that can predict pregnancy outcome?



Suha Kilani, currently a senior scientist at IVF Australia in Sydney, started as an embryologist in 1987 in Amman at one of the busiest clinics in the Middle East. After moving to Australia in 1998, she completed her Masters degree and recently gained her PhD from the University of New South Wales. In her thesis, supervised by Professor Michael Chapman, she investigated markers in human oocytes to better select the best embryo for transfer. She has presented her work at ESHRE and FSA Scientific Meetings.

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Abstract

Predictive criteria for selection of the best embryo for single embryo transfer remain elusive. This study aimed to determine if non-invasive markers in human oocytes, detectable using polarized light microscopy, can predict pregnancy outcome. Twenty-two pregnancy-producing oocytes from 19 patients had their morphological features compared with 30 oocytes from 19 age-matched patients whose transfer did not result in a pregnancy. Both pregnant and non-pregnant patients had similar numbers of oocytes collected (average: 11.9 ± 2.8 versus 11.3 ± 2.9) and similar fertilization rates (70.1% versus 69.6%). All embryos transferred were 4-cell cleavage-stage on day 2 with <10% fragmentation. Meiotic spindles were examined at 39–40 h following human chorionic gonadotrophin administration for spindle normality, length, density and angle from first polar body. There was a significant difference in spindle normality in oocytes in the pregnant patients compared with oocytes in the non-pregnant patients (100% versus 33%, $P < 0.001$). Spindle density was significantly higher in those oocytes resulting in pregnancy (3.0 ± 1.23 nm versus 2.5 ± 0.7 nm, $P = 0.02$). These oocyte markers may provide a useful non-invasive tool in the selection of the embryo most likely to produce a pregnancy.

Keywords: *ICSI, meiotic spindle, PolScope, pregnancy outcome*

Introduction

The selection of embryos with the highest implantation potential has been one of the major challenges in assisted reproduction. Initially, multiple embryo transfer was used to maximize pregnancy rates. However, improved embryo quality and rising multiple pregnancy rates has resulted in the reduction of the number of embryos to be transferred. Thus, selection of the 'best' embryo has become essential, particularly with elective single embryo transfer being strongly recommended or mandatory in a number of countries in women less than 35 years of age (Reproductive Technology Accreditation Committee, 2005).

Much research has focused on methods to define the best possible embryo for transfer. Survey of the literature shows that there have been many attempts to discover a non-invasive marker able to identify competent embryos (Kahraman *et al.*, 2000; Ebner *et al.*, 2003; Scott, 2003; Verlinsky *et al.*, 2003; Coticchio *et al.*, 2004; Kattera and Chen, 2004; Moriwaki *et al.*, 2004; Sun *et al.*, 2005; Scott *et al.*, 2007). Most of these have relied on visual markers of embryo development, which can be influenced by human bias, variability and low predictability (Blake *et al.*, 2002).

Other approaches to assess the embryo quality have been developed to measure end products produced in culture medium (Attaran *et al.*, 2000; Bedaiwy *et al.*, 2002; Houghton *et al.*, 2002). None of these techniques has been rapid enough to be used in a clinical setting. Studies involving invasive techniques have usually been performed in animal models, such as mice, and are not applicable to the human IVF process because of concerns of damage to embryos and long-term risks to fetal development. Natural selection of embryos by growing them for up to five days *in vitro* has been useful in improving pregnancy rates (Blake *et al.*, 2002).

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The introduction of polarized light microscopy (PolScope-LC) provides a non-invasive approach to the assessment of human oocytes and embryos. Using PolScope, non-invasive imaging and analysis of any birefringent object can be undertaken in the oocyte (i.e. zona pellucida, the meiotic spindle) (Wang and Keefe, 2002; Pelletier *et al.*, 2004). The PolScope has been used in a number of IVF laboratories to inject human oocytes, while viewing the meiotic spindle, in order to avoid damaging the spindle during sperm injection (Wang *et al.*, 2001; Cooke *et al.*, 2003). The results of these studies demonstrated that the embryo quality is significantly better (Cooke *et al.*, 2003). To date, of those studies that have used the PolScope, few have looked at the human oocyte nor defined markers to select the 'best' oocyte and then the 'best' embryo (De Santis *et al.*, 2005; Shen *et al.*, 2006; Rama Raju *et al.*, 2007).

As far as is known, this is the first study to look at non- invasive markers via polarized light microscopy in pregnancy-producing oocytes and comparing them with aged-matched non-pregnancy-producing oocytes.

Materials and methods

Trial design, inclusion criteria

Using the PolScope, oocytes from 211 patients were imaged at the time of intracytoplasmic sperm injection (ICSI) (39–40 h following administration of human chorionic gonadotrophin (HCG)). Injections were performed using the PolScope. The images were analysed prior to the knowledge of pregnancy outcome.

Pregnancies that fulfilled the criterion of every embryo transferred resulting in a pregnancy were selected as providing the oocytes for the pregnant group. Patients who did not conceive, but who fulfilled the criteria of producing high quality embryos, with a single or double embryo transfer, or at least one sibling embryo cryopreserved, and who were within 2 years of the age of a patient in the pregnant group were selected as the age-matched non-pregnant group. The selection of the non-pregnant group as having sibling embryos frozen in the same cycle was to ensure that the transferred embryos were selected from a group of high quality embryos.

Ovarian stimulation

Ovarian stimulation was achieved by a long-down regulation combination of a gonadotrophin-releasing hormone (GnRH)-agonist and FSH. The GnRH-agonist was either a subcutaneous injection (lucrin, leuprorelin acetate, Abbott, Kurnell, Australia) or a nasal spray (syneral, nafarelin acetate solution, Searle, Rydalmere, Australia). Recombinant FSH was used (Gonal-F, Serono, Frenchs Forest, Australia, or Puregon, Organon, Lane Cove, Australia) to induce follicular growth, and 10,000 IU of HCG (Profasi; Serono or Pregnyl; Organon) was administered to achieve final maturation and trigger ovulation when at least two follicles were at least 18 mm in diameter as measured by transvaginal ultrasound. Thirty-six hours later, ultrasound-guided oocyte retrieval was performed using a single lumen 17 G ovum pick-up set (Cook, Eight Mile Plains, Australia). All oocytes were returned to the IVF laboratory in HEPES-buffered medium (Sage, Bedminster, USA) at 37°C in a portable incubator (LEC-960, LEC Instruments, Scoresby, Australia).

Environment during oocyte injection, embryo culture and image analysis

Oocytes had their cumulus cells removed by 30 s exposure to 30 IU/ml hyalase (CP Pharmaceuticals, UK) in HEPES buffered medium (Sage) followed by washing with HEPES buffered human tubal fluid containing 5 mg/ml human serum albumin (Sage). The coronal cells were mechanically removed by use of 135 μl flexi-pipettes (Cook).

Denuded oocytes were then assessed for their maturity and the presence of the first polar body (1PB) and placed into culture in 0.8 ml fertilization medium covered with a pre-warmed tissue culture oil (Sage), in a pre-equilibrated 4-well nunc dish (Nunclon, Medos, Lidcome, Australia) that had been pre-equilibrated at 37°C in 5% carbon dioxide, 5% oxygen and 90% nitrogen. Oocytes were microinjected 39–40 h after the ovulatory HCG injection. ICSI was performed, using PolScope optics (Vital Diagnostics, Castle Hill, Australia), in a 5 μl droplet of HEPES-buffered medium in a sterile glass bottomed dish (Willco Wells, Amsterdam, The Netherlands). Oocytes were rotated four times around each axis until a full image of the meiotic spindle was obtained, then the image was captured. Captured images were saved for later analysis by the PolScope computer software.

Microinjection was performed 39–40 h after HCG administration (Kilani *et al.*, 2006) by holding the oocyte with the spindle in the 12 o'clock position and the injecting the needle into the oocyte at the 3 o'clock position (Cooke *et al.*, 2003). If an oocyte had a spindle 90° away from the 1PB, then the oocyte would be held with the spindle n the 12 o'clock position with the polar body at 9 o'clock while injecting at the 3 o'clock position.

Following microinjection and imaging, the oocytes were transferred to equilibrated individual numbered 20 μl droplets for single culture in fertilization media in a sterile plastic dish (Nunclon) (Cooke *et al.*, 2003).

All oocyte–embryo culture was performed in a mini incubator (MINC-1000, Cook) supplied with a humidified triple gas mixture of (5% carbon dioxide + 5% oxygen + 90% nitrogen) at 37ºC.

Fertilization was assessed 16–20 h (day 1) after microinjection. Fertilized oocytes were transferred to individual drops of HEPES buffered medium for image capture. Zygotes were then washed in their respective cleavage medium (Sage) and each zygote was placed in its respective numbered 20 μl droplet of medium for culture (Cooke *et al.*, 2003). Cleavage was defined as the presence of a 2- or 4-cell embryo approximately 40 h (day 2) after ICSI. The same imaging technique was used to capture embryo images for later analysis. A transcervical embryo transfer was performed 41–44 h after microinjection, using a double-lumen catheter (K-JETS, Cook). All patients received luteal phase supplementation of daily crinone 8% vaginal gel (Serono).

Spindle normality

Spindle view with Olympus IX70 microscope was used for image capture and analysis. Lens magnification used was ×40. For the purpose of this study, a spindle was considered as normal if it was complete, barrel shaped and showed strong birefringence (i.e. bright against a dark background) (**Figure 1**). Any other shape was considered abnormal (**Figure 2**).

Measurement protocol used to achieve spindle length and density

The spindle was measured using the PolScope computer software. The oocyte images were retrieved and the measurement for quantitative analysis of spindle length and density was performed by the same operator, blinded to the outcome of the patient.

A straight line scan along the entire spindle, from pole to pole was created (i.e. the scan line divided the spindle into two approximately equal parts). The line was then adjusted, using the computer mouse, for measurement accuracy, starting with the outer pole and when the retardance was equal to the background and ending with the inner pole until the retardance dropped down to equal the cytoplasm (**Figure 3**). All measurements, for spindle length recorded in μm and spindle density in nm, were repeated four times and the average of the four measurements was used to ensure accuracy of data analysis. The choice of four measurements was arbitrary.

Measurement of the angle between the spindle and 1PB

The angle of the meiotic spindle from the 1PB was measured by assigning three points on the oocyte. The first point started at the 1PB, the second was at the middle of the oocyte and the third ended at the meiotic spindle. The angle was measured by degrees and calculated by the PolScope computer software (**Figure 4**).

Study end-points

The end-points of the study were the detection of a normal meiotic spindle, measurements of spindle density, spindle length and the angle from the 1PB in mature oocytes.

Statistical analysis

The chi-squared test was used to compare the percentages of normal meiotic spindles detected in the pregnant and non-pregnant group of oocytes. Two-tailed Student's *t*-test was used for quantitative analysis of spindle retardance, spindle length and its angle from 1PB. All data analysis was performed using Statistics Package for Social Sciences (SPSS) software (SPSS, USA).

Approval for the study was obtained from the IVF Australia National Health and Medical Research Council and the Human Research Ethics Committee.

Results

A total of 1477 oocytes from 211 patients (age range: 20–48 years) were imaged at the time of ICSI (39–40 h post HCG) using the PolScope. All 1477 oocytes were injected using the PolScope. Of those, 1265 (86%) had a visible spindle at the time of injection. The images were analysed prior to the knowledge of pregnancy outcome.

Forty-four patients became pregnant and 19 pregnancies fulfilled the criterion of every embryo transferred resulting in a pregnancy ($n = 22$) (pregnant group).

Of the 167 patients who did not become pregnant, 28 had embryo(s) transferred that in the same cycle also had embryos frozen. Nineteen of those patients fulfilled the inclusion criterion of having at least one sibling embryo cryopreserved but did not conceive, and were within 2 years of the age of the matched pregnant group. This group of transferred embryos ($n = 30$) provided the non-pregnant group of oocytes.

The two groups comprised oocytes from patients of comparable maternal age, average number of oocytes retrieved (11.9 ± 2.8 versus 11.3 ± 2.9) and fertilization rates (70.1% versus 69.6%). The quality of all embryos transferred in both groups scored 4A (4 cells on day 2 with <10% fragmentation) which are considered suitable for freezing or transferring in the IVF Australia scoring system.

Meiotic spindle analysis

Twenty-two oocytes (19 patients) from the pregnant group were analysed and compared with the 30 oocytes from the non-pregnant group (19 patients).

Spindle normality

100% (22/22) of the pregnancy group and 100% (30/30) of the non-pregnant group had a visible spindle at 39–40 h post HCG. When analysed for spindle normality 22/22 (100%) of the pregnancy-producing oocytes had normal spindles whereas only 10/30 (33%) oocytes in the non-pregnant group had normal spindles ($P < 0.001$) (**Table 1**).

Spindle retardance

The average spindle retardance in the pregnant group was significantly higher than in the non-pregnant group (retardance ± SD: 3.0 ± 0.99 nm versus 2.5 ± 0.7 nm, $P = 0.02$; **Table 1**).

Spindle length

Spindle length in the pregnant group was shorter than in the non-pregnant group but this difference was not significant (18.6 ± 2.3 μm versus 19.4 ± 2.3 μm).

Angle from the 1PB

There was no significant difference in the spindle angle from the 1PB between the pregnant and non-pregnant group (22.5 ± 28.13° versus 22.5 ± 19.0°).

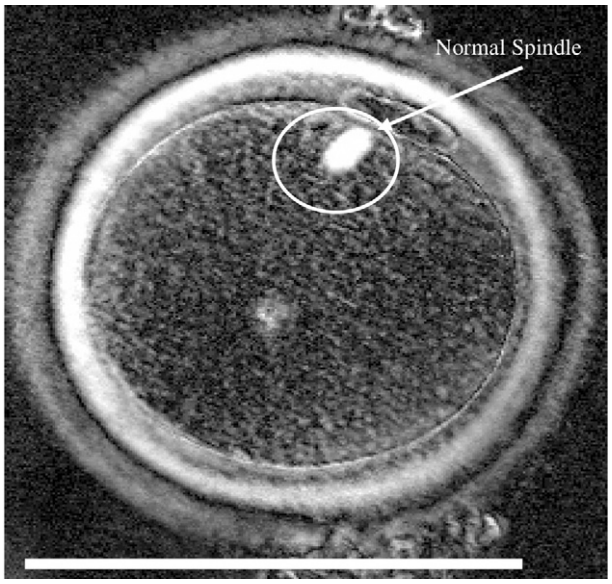


Figure 1. An oocyte with a normal meiotic spindle: complete, barrel shaped and birefringent (bar = 100 μm).

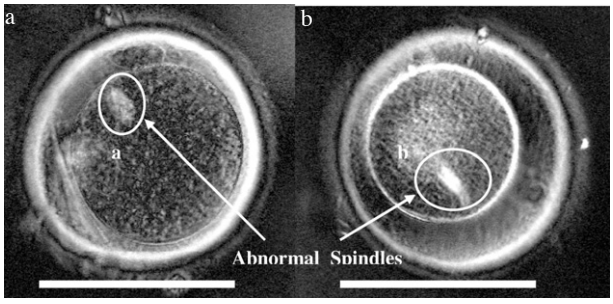


Figure 2. Oocytes with abnormal spindles (bar = 100 μm): (**a**) partial and (**b**) non-barrel-shaped spindles.

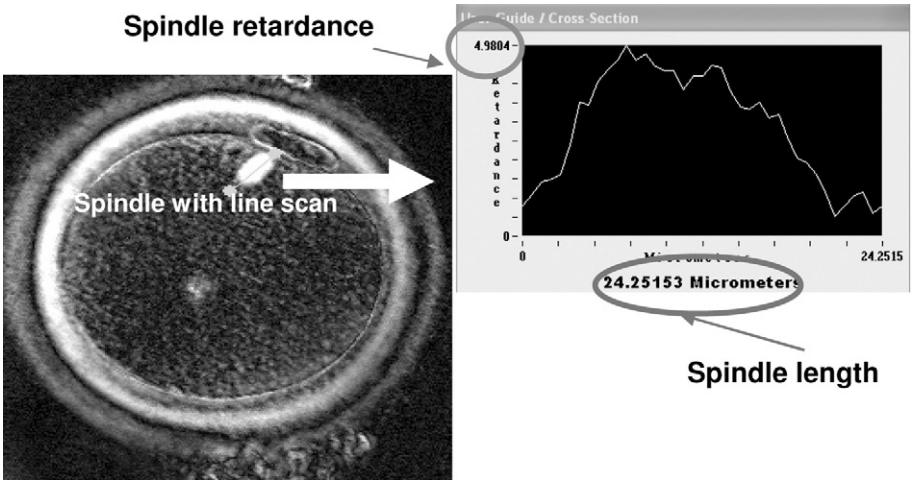


Figure 3. Method used for measuring spindle length and retardance. Using the PolScope computer software, a line scan was pulled from pole to pole along the spindle. The peak of the graph drawn was taken as the spindle retardance.

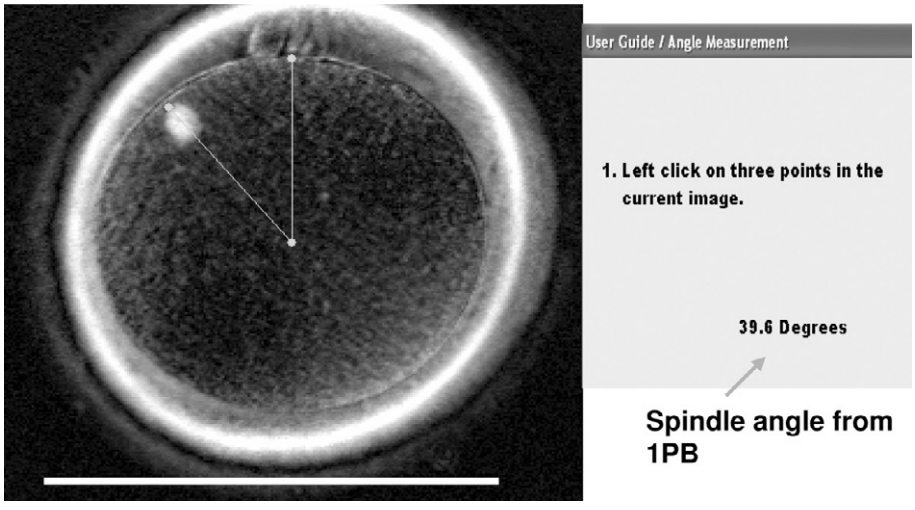


Figure 4. Measurement of spindle angle from the first polar body (1PB): 39.6° in this image (bar = 100 μm).

Table 1. Meiotic spindle analysis. Comparison between oocytes that generated pregnancy embryos and non-pregnancy embryos.

Parameter	*Pregnant group*	*Non-pregnant group*
No. of MII oocytes	22	30
No. of visible spindles (%)	22/22 (100)	30/30 (100)
No. of normal spindles (%)	22/22 (100)[a]	10/30 (33)[b]
Maximal spindle retardance (nm)	3.0 ± 0.99[c]	2.5 ± 0.7[d]
Spindle length (μm)	18.6 ± 2.3	19.4 ± 2.3
Angle from first polar body (°)	22.5 ± 28.13	22.5 ± 19.0

Values are mean ± SD unless otherwise stated. MII = metaphase II.
[a,b]$P < 0.001$; [c,d]$P = 0.02$.

Discussion

The quest for a non-invasive method of assessing the embryo with the highest potential to produce a pregnancy continues to frustrate scientists, clinicians and patients. This pursuit has heightened over the last 5 years as elective single embryo transfer has become more common.

Polarized light microscopy has increased the number of morphological features of the oocyte that can readily be assessed non-invasively. This technique may reveal aspects of the oocyte that demonstrate its competency.

One deficiency, apparent in the literature, is a lack of any significant validation of the PolScope measurement techniques both in terms of standardized timing of observations, given the known dynamic state of the oocyte, as well as the reliability and reproducibility of the measurement techniques. The uniqueness of this study is that the analysis techniques have previously been validated and standardized (Kilani *et al.*, 2006).

In this study, the spindle in the oocytes of pregnancy-producing embryos have been compared with an age-matched control group of oocytes that did not result in pregnancy. To ensure that this control group had oocytes of equivalent quality, patients were only included if they had sibling embryos frozen in that cycle. In the study laboratory, embryos are only frozen if they have 4 cells on day 2 and <10% fragmentation. As it transpired, when standard morphological criteria of cell number and degree of fragmentation were compared, the quality of pregnancy-producing and non-pregnancy-producing embryos was similar.

This study showed that the presence of any spindle (normal or abnormal) was high in all oocytes that produced good quality embryos, whether they produced a pregnancy or not. However, in pregnancy-producing oocytes, 100% had normal spindles whereas in those oocytes that did not result in a pregnancy only one-third had normal spindles. This proportion in non-pregnancy-producing oocytes is substantially lower than the overall normal rate of 55% previously reported (Kilani *et al.*, 2006). Thus, on the basis of this study, oocytes that fertilize and develop to apparently high quality cleavage-stage embryos appear to have a lower chance of success in achieving a pregnancy if they do not possess a normal meiotic spindle. The important corollary of this is that the presence of a normal meiotic spindle seems to be associated with a high pregnancy potential and so its existence may be a useful when deciding which is the best embryo for transfer after ICSI. Extrapolation of these results would suggest that when a normal spindle is found, the chances of a pregnancy, if a high quality cleavage stage embryo follows, is greater than 65%.

Previous studies showed that oocytes with absent or abnormal spindle have decreased fertilization rates (Wang *et al.*, 2002). These oocytes, if fertilized, have a lower overall capacity to develop into a normal embryo (Shen *et al.*, 2006). The results of the present study showed that oocytes with an abnormal spindle can fertilize and develop into an apparently high-quality embryo. It also confirms that on standard light microscopy criteria it is not possible to predict accurately a pregnancy-producing embryo.

The finding that apparently high-quality embryos can be created, but their capacity to produce a pregnancy cannot be differentiated via light microscopy, has been confirmed in preimplantation genetic diagnosis studies. These studies showed that genetically abnormal embryos can grow to day 5 and 6 blastocysts and cannot be differentiated from normally developing embryos (Baltaci *et al.*, 2006; Rubio *et al.*, 2007).

The classification of the 'normality' of the spindle is relatively subjective. To exclude observer bias, the analyses of the oocyte parameters in this study were performed prior to the knowledge of the pregnancy outcome. The technique used has been assessed as having a relatively low coefficient of variation prior to undertaking the study. However, for this assessment to be used in the future, a more specific qualitative definition of spindle normality needs to be developed.

Spindle retardance has been suggested as a further marker of embryo quality. This has been assessed in few studies of human oocytes. These have shown that the increased spindle retardance reflects high embryo quality (Liu *et al.*, 2000; De Santis *et al.*, 2005). De Santis *et al.* (2005) reported a possible correlation between spindle retardance of oocyte and embryo quality. However, neither study standardized for the timing of observation, which could be critical, since the spindle is a dynamically changing structure. The present results concur with these reports. Statistically lower spindle retardance in the

non-pregnant group was observed in this study. However, the overlap in the results of spindle retardance in the two groups would suggest that this measurement has limited use in selecting the 'best' embryo. In the current study, the average maximal spindle retardance was used, however, another approach would be to compare the mean spindle retardance using all the measurement points along the whole line scan. It would be interesting to compare the two methods in terms of reproducibility and predictability on oocyte quality in further studies.

The position of the spindle in relation to the 1PB was similar in the two groups and appears to have no influence on embryo development. This agrees with Moon *et al.*, (2005) who showed that the relative position of the spindle within the oocyte does not appear to influence the developmental potential of embryos. In another study, the meiotic spindles of in-vitro matured oocytes were analysed, and found to be aligned with the 1PB. This suggests that the misalignment observed in the oocytes matured *in vivo* results from 1PB displacement during cumulus and corona removal (Rienzi *et al.*, 2005).

Spindle length has been studied previously and related to embryo development (Rama Raju *et al.*, 2007). These authors showed that more blastocysts were obtained from oocytes with spindle lengths of >12 nm than from oocytes with spindle lengths 10–12 nm or <10 nm. In the present study, spindle length was shorter in pregnancy oocytes compared with non-pregnancy oocytes. However, the spindle length in the pregnancy-producing oocytes was comparable to that in the study of Rama Raju *et al.* (2007). This might suggest that there is an upper and lower limit for a spindle to be considered as normal. Further studies are needed to confirm this finding. The standardization of the imaging and measurement techniques and their timing at a fixed interval (39–40 h post HCG) adds strength to the observations and the conclusions drawn. It may be that timing of observations is critical since time-course studies have revealed that most spindles are visible at 39–40 h post HCG and are significantly less visible in the hour either side of this peak (Kilani *et al.*, 2006).

This study has shown a lower pregnancy rate than that mentioned in the literature (Konc *et al.*, 2004). The overall pregnancy rates, at the same laboratory where this study were performed, were 35% for patients ≤38 years old. The reason for this lower pregnancy rate may be the quality of patients involved in the study. The majority of patients had been referred for PolScope for repeated IVF failure.

In conclusion, this study suggests that the presence of a normal spindle in an oocyte that produces a good quality embryo on day 2 after ICSI is a major predictor of the likely success of that embryo. Together with spindle retardance, the detection of a normal spindle with polarized light microscopy provides non-invasive markers that may assist in improving pregnancy rates in assisted reproduction treatment. The clinical implication of this finding is that if one embryo is to be transferred, and two or more good quality embryos have been generated, the choice of which embryo to use should be made on the presence of a normally shaped spindle in the initiating oocyte. This concept needs to be tested in a properly conducted randomized controlled trial.

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