Reproductive BioMedicine Online (2010) **20**, 639–648

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ARTICLE

Spindle and chromosome configurations of human oocytes matured *in vitro* in two different culture media

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Abstract In-vitro maturation can have deleterious effects on spindle formation and proper chromosome alignment in human oocytes and can be profoundly affected by culture conditions. This study compared the spindle presence and location with the maturation rate of germinal vesicle (GV) oocytes cultured in two different media: G1.2 and G1.2 supplemented with follicle-stimulating hormone, human chorionic gonadotrophin and 17β-oestradiol. A total of 304 oocytes were retrieved from 101 women undergoing IVF treatment with intracytoplasmic sperm injection. Spindle presence was recorded using the Polscope. Spindle morphology was evaluated with immunocytological staining for α-tubulin and chromatin. Twenty-one in-vitro matured oocytes with the presence of spindle and ten of their corresponding polar bodies (PB) were also assessed for aneuploidy. A significantly increased maturation rate (69.7%) was observed after 24 h in the supplemented culture media compared with the G1.2 media (56.6%; $P < 0.05$). The proportions of metaphase II (MII) oocytes with spindle presence and abnormal spindle morphology were similar in the two culture media. Also, 76.9% of MII and 70% of PB had chromosomal abnormalities. In conclusion, supplementing culture media may increase the oocyte maturation rate *in vitro*, but does not necessarily indicate the presence of a birefringent spindle, or normal spindle and chromosomal alignment.

KEYWORDS: culture medium, FISH, immunostaining, oocyte maturation, polscope, spindles

Introduction

In-vitro maturation (IVM) of human oocytes was first demonstrated by Edwards in 1965 (Edwards, 1965), preceding by more than a decade his first successful IVF attempt in 1978 (Steptoe and Edwards, 1978). Since then, IVF has followed different pathways, which have led it far from the necessity of IVM. Assisted reproduction techniques

1472-6483/$ - see front matter
doi:10.1016/j.rbmo.2010.02.005

became more effective and the stimulation protocols provided many alternatives using either gonadotrophin-releasing hormone (GnRH) agonists for pituitary suppression in long or short protocols (Kerin, 1989), or GnRH antagonists (in order to prevent premature LH surge) which provide a patient-friendly solution (Al-Inany et al., 2006).

Lately there is increasing demand for less drug-oriented, lower risk, less expensive and more patient-friendly approaches to assisted reproduction, embracing gentle stimulation protocols and IVM of oocytes. Extensive research plus the latest pregnancies successfully achieved in Canada after IVM (Al-Sunaidi et al., 2007) suggest the usefulness and the need for improvement of this technique.

This renewed interest is of great importance for women with polycystic ovary syndrome who are classified as high responders to ovarian stimulation protocols. Their vigorous response to stimulation may lead to ovarian hyperstimulation syndrome, which in its severe forms leads to cycle cancellation, hospitalization and, in rare cases, death (Enskog et al., 1999; Rizk, 1995). IVM seems to be a viable alternative for these women, since the milder stimulation protocol required in this procedure is expected to significantly reduce the risk of ovarian hyperstimulation syndrome.

However, if the outcome measures in the specific subgroup should become comparable to classic protocols, then it is understandable that they could soon encompass a wider population of women undergoing IVF, since protocols including IVM would lead to milder and more simplified ovarian stimulation protocols and a reduction in the cost of treatment (Jurema and Nogueira, 2006). There are two other scenarios where improvement of IVM technology will prove beneficial. The first encompasses the salvage of immature oocytes for IVF (intracytoplasmic sperm injection; ICSI) after standard stimulation and the second involves fertility preservation with the cryopreservation of oocytes matured *in vitro*. The potential of this technology to restore fertility in women anticipating sterility secondary to cancer treatment is an important and exciting prospect (Elizur et al., 2007; Huang et al., 2008).

Recent clinical results from in-vitro matured human oocytes are promising (Chian et al., 2003). However, the developmental and implantation potential of IVM oocytes have been reported to be low (Mikkelsen et al., 1999). It has been suggested that the reduced developmental potential in human oocytes matured *in vitro* may be attributable to sub-optimal culture conditions, incomplete oocyte growth or abnormal cytoplasmic maturation and distortion of spindle apparatus (Moor et al., 1998). Disruption of spindle morphology leads to chromosome abnormalities that are reported to be high in human oocytes, especially among those matured *in vitro* (Gras et al., 1992; Racowsky and Kaufman, 1992).

A major technological innovation that gave a tremendous boost to IVM studies is the Polscope, which allows the non-invasive study of spindles during the maturation of oocytes (Liu et al., 2000). It was established that the spindle images obtained with the Polscope in living human oocytes are coordinate with those in fixed oocytes as imaged by confocal microscopy (Wang and Keefe, 2002). By using the Polscope, it was found that the presence of a birefringent meiotic spindle in human oocytes could predict a higher embryonic developmental competence (Moon et al., 2003) and a higher fertilization rate (Wang et al., 2001a,b). However, the relative position of the spindle within the oocyte did not appear to influence the developmental potential of embryos (Moon et al., 2003).

The most important factor affecting oocyte maturation *in vitro* is the culture conditions and specifically the composition of the culture media. Although numerous data have been accumulated from animal studies, the current rationale for choosing a specific medium for IVM of immature oocytes appears to stem largely from adapting methods developed from culturing other cell types (Chian et al., 2003). The most common culture media used in IVM are G1, IVF20 and CCM supplied by Vitrolife (Goterborg, Sweden) and Medicult (Mollehaven, Denmark). The composition of culture media used successfully for maturation of human oocytes is surprisingly similar to that originally developed for maturation of oocytes in follicle culture *in vitro*. The presence of follicle support cells in culture is necessary for the gonadotrophin-mediated response required to mature oocytes *in vitro*. Gonadotrophin concentration and the sequence of follicle-stimulating hormone (FSH) and FSH/LH exposure may be important for human oocytes, particularly those not exposed to the gonadotrophin surge *in vivo* (Trounson et al., 2001).

The aim of the present study was to compare the maturation rate of germinal vesicle (GV) oocytes cultured in two different media and to record the spindle presence, location (deviation angle) and morphology as well as the chromosome abnormalities.

Materials and methods

Source of oocytes

The study was approved by the local institutional review board. A total of 304 oocytes at the GV stage were retrieved from 101 consenting patients undergoing the ICSI programme in the study centre between January and December 2006. The patients' ages ranged from 29 to 43 years, with a mean ± SD of 33.2 ± 2.5 years. Patients were allocated to the study when a minimum of 10 metaphase II (MII) oocytes and two oocytes at the GV stage were obtained after retrieval. **Table 1** summarizes the aetiology of infertility of patients included in the study and the clinical outcome of their stimulation cycles.

Monitoring and oocyte preparation

Ovarian stimulation was achieved by a long down-regulation with a GnRH-agonist and recombinant FSH, either with Gonal-F (Merck Serono Europe, London) or Puregon (Organon, Oss, Holland) to induce follicular growth. The mean ± SD of the starting dose of recombinant FSH was 345 ± 134 (IU) and for total dose was 3150 ± 950 (IU) during stimulation cycles. When three leading follicles were >18 mm in diameter, 10,000 IU human chorionic gonadotrophin (HCG) (Pregnyl, Organon) was administered, 36 h before oocyte collection. Oocyte–cumulus-complexes were obtained via ultrasound-guided transvaginal aspiration.

Cumulus and corona cells were removed from oocytes by exposure to HEPES-buffered medium (Sage IVF, Thumbull, CT, USA) containing 40 IU/ml hyaluronidase (IV-S, H4272;

Table 1 Patient characteristics and cycle outcome data.

Parameter	*Value*
No. of patients	101
No. of cycles	101
Maternal age (years)	33.2 ± 2.5
Cause of infertility	
Male factor	62
Tubal factor	10
Endometriosis	5
PCO	5
Combination of two or more	18
MII oocytes per patient	12.1 ± 2.6 (10—19)
GV oocytes per patient	3.1 ± 0.9 (2—5)
Fertilization rate	981/1212 (81)
Clinical pregnancy rate/oocyte retrieval	41/101 (41)
Implantation rate	61/210 (29)

Values are mean ± SD, (%), mean ± SD (range) or number/total (%) unless otherwise stated.
GV, germinal vesicle; MII, metaphase II; PCO, polycystic ovaries.

Sigma, USA) and by using fine glass Pasteur pipettes. Meiotic maturation state of the oocytes was then assessed. Oocytes with first polar body appearance (presumably at the MII stage) were immediately fertilized by ICSI. Oocytes without first polar body extrusion and those with the appearance of GV were matured *in vitro*.

IVM procedure

After denudation, an even number of GV oocytes from each patient was randomized using a random numbers table into two groups: group A: 152 oocytes cultured with G1.2 medium (Vitrolife, Sweden); group B: 152 oocytes cultured with G1.2 medium supplemented with 0.075 IU FSH (Gonal-F; Merck Serono, Switzerland), 0.05 IU HCG (Profasi; Merck Serono) and 1 μg 17,β-oestradiol (Sigma). G1.2 medium contains EDTA (antioxidant) essential and non-essential amino acids, human serum albumin (HSA), inorganic salts, pyruvate, lactate and glucose.

The oocytes were cultured in groups of four in droplets of 50 μl of culture media with an oil overlay (Irvine, USA) in pre-equilibrated Falcon dishes (353652; BD, Falcon). Oocytes achieved nuclear maturation and reached MII stage with the extrusion of first polar body as examined under the stereomicroscope (SMZ-1500, Nikon) 24 h after culturing. Evaluation of oocyte maturation was not carried out over a longer period of time than 24 h.

Spindle identification of in-vitro matured oocytes with the Polscope

Meiotic spindle was observed in MII oocytes and oocytes at post-GV stages after IVM using the Polscope spindle view system (LC-Polscope Pro version 3.9; CRI, Woburn, MA, USA) optics controller at 20× magnification combined with a computerized image analysis system (SpindleView, CRI). The oocytes were placed in a 50 μl drop of MOPS and HSA and covered with mineral oil (Irvine Scientific, Santa Ana, USA) in a glass-bottom culture dish (Willco Wells, Amsterdam, Netherlands), which was maintained at 37°C on a heated stage (LEC Instruments, Australia). For this purpose, the oocyte was immobilized with a holding pipette and rotated with the injection pipette until both the meiotic spindle and polar body were visible. In oocytes where the spindle was visible, the deviation angle was measured in relation to the polar body position. This was accomplished by drawing two imaginary lines, one through the centre of the oocyte and the polar body and the other at an angle of 90°. The oocytes were rotated perpendicularly around these axes until the spindle was close to the cortex. Angle measurements were made with the spindle close to the cortex of the oocyte (**Figure 1**).

Oocyte fixation and immunofluorescence staining

Oocytes that had reached the MII stage were immunostained for tubulin and chromatin detection using a protocol modified from Pickering et al. (1988) after Polscope analysis. Oocytes were fixed individually in 3.7% formaldehyde/phosphate-buffered saline (PBS) (Sigma) and permeabilized with 0.05% Triton X-100 in PBS overnight, then incubated in blocking solution (PBS with 2 mg/ml and 150 mmol/l of glycine) for 30 min followed by anti-β-tubulin monoclonal antibody in PBS (1:300; Sigma) for 1 h at 37°C and subsequently washed in goat anti-mouse immunoglobulin G (1:5000; Boehringer Manheim Biochemica, Germany) in PBS/Tween 20 for 1 h

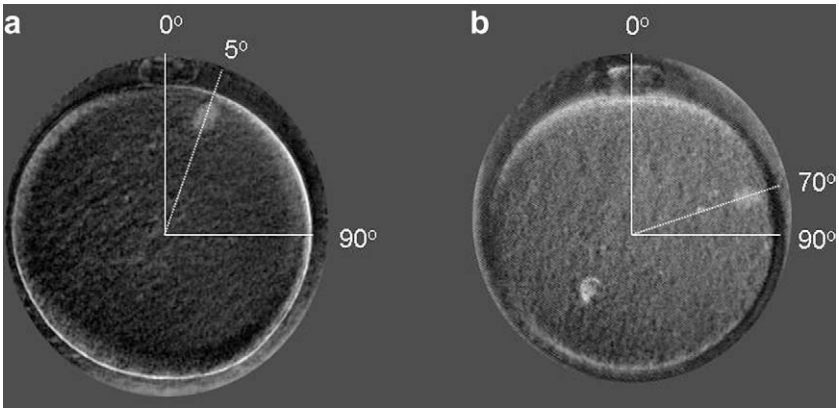

Figure 1 Schematic representation of the spindle deviation angle compared with the polar body position, measured in the equatorial plane of the oocyte. The line between the centre of the oocyte and the polar body is set at 0° while the line at an angle of 90° is used to estimate the angle between the visualized spindle and the polar body. **(a)** Deviation angle of 5° between the spindle and the PB position; **(b)** Deviation angle of 70° between the spindle and the PB position. Magnification 20×.

and FITC conjugated with extravidin (1:50; Sigma) for 30 min in order to stain the microtubules. Finally, the chromatin was counterstained with DAPI (0.5 μg/ml in antifade solution; Sigma) by incubating the oocytes for 10 min. Between incubations, the oocytes were washed in Dulbecco's PBS prewarmed at 37°C three times for 5 min. Samples were then mounted on SuperFrost slides (SuperFrost plus; VWR, USA) with antifade solution (Vectashield; Vector Laboratories Inc., USA) to avoid photo bleaching. Labelled tubulin and chromatin were assessed using an epifluorescence microscope (Eclipse E800; Nikon) equipped with the appropriate filter sets for analysing FITC and DAPI with 60× oil immersion objective. Oocyte images were captured with Spot Insight digital cooled charge-coupled device (CCD) camera and analysed using a data-imaging program (Image-Pro Plus software; Media Cybernetics, Maryland, USA, 2000). The images were then compared with those obtained from Polscope.

MII chromosome preparation

Chromosome spreads were prepared from 21 IVM MII oocytes with a visible spindle and an abnormal morphology. Gradual fixation (Angell et al., 1994) was used for this purpose. Each oocyte was treated with a hypotonic solution of 0.9% sodium citrate and 0.6% BSA (Sigma) at 37°C for 15 min. It was then transferred to an embryo culture dish containing fresh fixative I (methanol/acetic acid/distilled water, 5:1:4, v/v). The fixative gradually dissolved the zona pellucida. By using a pulled pipette the oocyte was transferred on a SuperFrost slide rinsed previously in pure methanol. As soon as the oocyte settled on the slide, a flow of fixative II (methanol/acetic acid, 3:1) followed. The slide was immediately placed in a coplin jar containing fixative II for 5 min and then to a second coplin jar containing fixative III (methanol/acetic acid/distilled water, 3:3:1, v/v) for 1 min to soften the cytoplasm. Finally, the slide was air-dried. Fixed oocytes were dehydrated sequentially through an ethanol series (70%, 85% and 100%) and examined under an inverted microscope (10× and 20× magnification; Olympus, CK30) to check the chromosome preparations.

Polar body biopsy and fixation

Biopsy of 10 polar bodies was performed mechanically (Verlinsky and Kuliev, 2005) by using the Zilos TK Laser. Each polar body was extracted with a biopsy micropipette (Humagen, Chalrotessville, VA, USA) and placed in a drop of water on a SuperFrost slide. The water drop was air-dried and two drops of Carnoy's solution (methanol/acetic acid, 3:1) were added.

FISH protocol

Fluorescence in-situ hybridization (FISH) was performed on 21 MII oocytes and on 10 of their corresponding polar bodies (PB), from both groups (A and B) with a visible spindle of an abnormal shape. This subset of oocytes was chosen for FISH analysis in order to assess efficiency of the technique in the laboratory as a preliminary test analysis.

FISH was carried out with the analysis of only two chromosomes since there was a limitation on the band pass filters that could be used. Commercially available probes used for chromosome 15 (CEP 15, D15Z1, alpha satellite, Spectrum Green; Vysis, Downers Grove, IL, USA) and chromosome 16 (CEP 16, D16Z3, satellite II DNA, Spectrum Orange; Vysis). Chromosome 16 was analysed since its trisomy mostly arises from MI non-disjunction (Hassold et al., 1995; Nicolaidis and Petersen, 1998) and is the commonest among trisomies of first-trimester miscarriages (Carp et al., 2001). Chromosome 15 was chosen since it is lethal in a trisomy and occurs as a result of meiosis I non-disjunction leading to a conception that is usually aborted spontaneously in the first-trimester of pregnancy.

The probe/hybridization mixture was added on the slide under a 20 × 20 glass coverslip (631—0122; VWR) and sealed

Table 2 Spindle visualization of oocytes after in-vitro maturation in G1.2 media (group A) and G1.2 supplemented media (group B).

Oocyte	*Group A*	*Group B*
GV oocytes	152	152
MII oocytes after 24 h	86	106
MII with visible spindle	49 (57.0)	58 (54.7)
MII oocytes with spindle from GV oocytes	32.2	38.2
Oocytes at post-GV stages after 24 h with spindle	2/18 (11.1)	3/32 (9.4)

Values are number, number (%) or %.
GV, germinal vesicle.
There were no statistically significant differences between the two groups.

with cow gum. The target and probe DNA were co-denatured at 73°C for 5 min and hybridized overnight at 37°C using the HYBrite (denaturation/hybridization system; Vysis) device. The slides were then washed in 0.4× saline sodium citrate (SSC)/0.3% NP-40 at 73°C for 3 min and in 2× SSC/0.1% NP-40 at room temperature for 1 min. Chromosomes were counterstained with DAPI (0.125 μg/ml) diluted in antifade solution (Vectashield) and signals were evaluated under epifluorescence microscope (Eclipse E800, Nikon), at 60× oil immersion objective magnification, equipped with a dual-band pass filter for the simultaneous observation of the spectrum orange and spectrum green signals. Images were captured with Spot Insight digital cooled CCD camera and analysed using Image-Pro Plus software (2000; Media Cybernetics, Maryland, USA).

Statistics

All outcome frequency measures (MII oocytes after 24 h of culture, MII oocytes with/without visible spindle, spindle location and morphology) were compared between the two groups using the chi-squared test. Statistical significance was set at 0.05.

Results

In-vitro maturation rates

In the supplemented group B, 152 immature oocytes matured *in vitro*. After 24 h of culture, 106 oocytes reached the MII stage, representing a maturation rate of 69.7% (106/152). Of the remaining oocytes, 28 (18.4%) were at the GV stage and 18 (11.8%) at post-GV stages. In the non-supplemented group A, the maturation rate (56.6%; 86/152) of immature oocytes was significantly lower ($P = 0.024$) compared with group B. Of the remaining oocytes, 34 (22.4%) were at the GV stage and 32 (21.1%) at post-GV stages.

Spindle visualization

Spindle was examined under the Polscope in all of the oocytes matured *in vitro* in both groups. A birefringent spindle was observed in 49 (57.0%) and 58 (54.7%) of MII oocytes from groups A and B. The percentage of oocytes at post-GV stage, after the maturation process, with a visible spindle is presented in **Table 2**.

Spindle deviation angle

Of the 86 and 106 oocytes matured *in vitro* in groups A and B, it was possible to determine the spatial relationship between the spindle and the first PB only in those with a visible spindle (49 in group A and 58 in group B). In group A, 91.8% of MII oocytes had their spindle at a close proximity to the PB (deviation angle of 0—5°) while 8.1% had a deviation angle more than 5° in the same or the opposite hemisphere. In the supplemented media group (B) 63.8% of MII oocytes had their spindle located close to the PB (angle 0—5°) while 36.2% of the remaining oocytes had a deviation angle of more than 5° in the same or the opposite hemisphere. The difference between the two groups was statistically significant ($P = 0.013$).

Spindle morphology

Spindle configuration was regarded as normal when a barrel-shaped structure with poles formed by organized microtubules traversing from one pole to the other as visualized under epifluorescence microscopy (Sathananthan, 2003) (**Figure 2**a). Chromosomal organizations were regarded as normal when they were aligned on a compact metaphase plate at the equator of the structure (**Figure 2**a). Abnormal spindle structure included disorganization of the microtubule fibres (**Figure 2**b and c). Abnormal chromosomal alignment included dispersion of chromosomes (**Figure 2**c).

The percentage of MII oocytes with a visible spindle and an abnormal morphology was similar in groups A and B (**Table 3**). Epifluorescence microscopy confirmed the spindle presence in all the MII oocytes with a birefringent spindle as revealed under polarized microscopy. On the other hand, immunofluorescence showed that a subset of oocytes without a visible spindle through Polscope analysis possessed a spindle but with an abnormal structure in both groups (**Table 3**). Microtubule fibres were absent in the remaining oocytes (group A: 19, group B: 23).

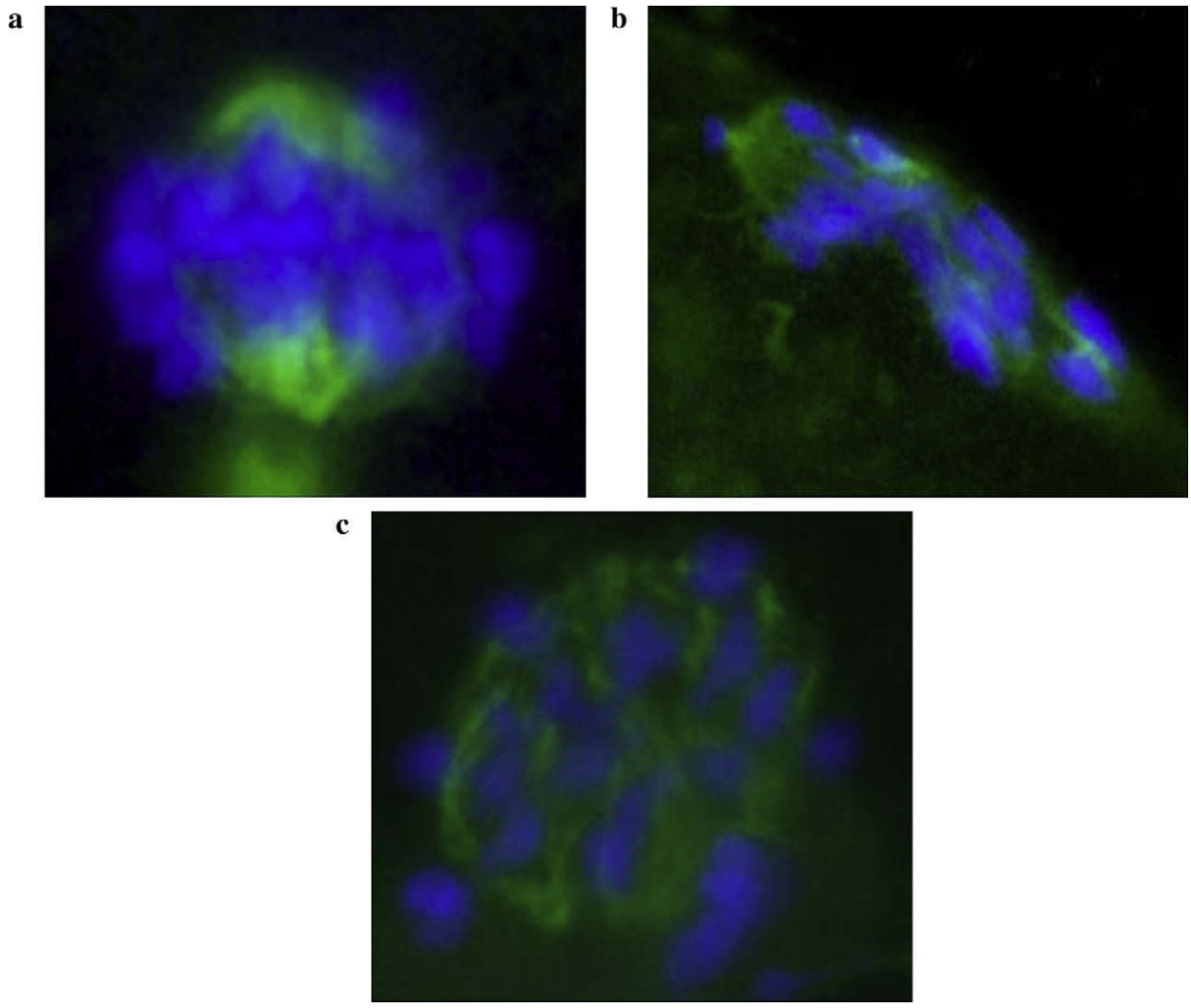

Figure 2 Epifluorescence microscopy images of spindle configurations with microtubules. **(a)** Equatorial view of normal spindle configuration with chromosomes aligned along the plate of the metaphase plane displaying a barrel-shaped spindle structure, magnification 100×; **(b)** Planar view of abnormal spindle configuration with chromosomal organizations associated with total disorganization of the microtubule fibres displaying an elongated spindle structure, magnification 100×; (c) Planar view of abnormal spindle configuration with dispersed chromosomal organizations associated with abnormal organization of the microtubule fibres displaying a round spindle structure, magnification 100×. Green = anti-α-tubulin monoclonal antibody and FITC; blue = DAPI.

FISH results

Preliminary results showed that the aneuploidy rate was higher compared with polyploidy both in PB and MII oocytes. The distribution of numerical chromosomal abnormalities is presented in **Table 4**. The first polar body is the mirror image of the oocyte, so the presence of double-dotted signals (one dot per chromatid) indicates no errors at meiosis I. Alternatively, the presence or absence of two additional dots implies that the oocyte is nullisomic or disomic respectively, while the presence or absence of a single-dotted signal (**Figure 3**) indicates the occurrence of a meiotic error due to the pre-division of chromatids.

Out of 10 PB, 7 (70%) matched their corresponding MII oocyte in terms of chromosome abnormalities. However, there was difficulty in obtaining all of the corresponding PB for the MII oocytes due to loss in PB fixation. It must be noted that these results are preliminary. Further analysis should include more chromosomes and should be applied to a bigger sample size.

Discussion

The meiotic spindle has been extensively studied over previous years as a possible predictive factor for oocyte quality. Its morphology and location are influenced by various factors such as maternal age, temperature, in-vitro manipulation and culture media. This study compares the difference in spindle presence, location and morphology along with the maturation rates of GV oocytes between

Table 3 Spindle visualization, location (relative to the polar body) and morphology of in-vitro matured oocytes in G1 (group A) and G1-supplemented media (group B).

Morphology	*Group A*	*Group B*
MII with visible spindle	49	58
Abnormal spindle morphology	13	14
MII with abnormal spindle morphology	26.5	24.13
MII with normal spindle morphology (within MII and with visible spindle)	73.5	75.87
Oocytes with spindle deviation angle of 0—5°	45	37
MII oocytes without visible spindle through polarized microscopy	37	48
MII without visible spindle and abnormal morphology through epifluorescence microscopy	18 (48.6)	25 (52.1)

Values are number, (%) or number (%).
There were no statistically significant differences between the two groups.

two different culture media. Nuclear and cytoplasmic maturation of the oocyte is influenced by different components in the media (Filali et al., 2008; Jurema and Nogueira, 2006). In the present study, the maturation medium used was G1.2 (either supplemented with gonadotrophins or not), which covers the basic metabolic needs of the oocyte. Specifically it contains amino acids that contribute to protein synthesis, HSA that is a source of albumin and growth factors, along with inorganic salts to maintain the osmolarity and pH. In addition, pyruvate, lactate and glucose are also included in the media and act in a complementary way as substrates for energy source. According to Haekwon and Schuetz (1991) pyruvate can directly enhance nuclear maturation of denuded oocytes whereas glucose needs to be metabolized by cumulus cells in response to LH (Zuelke and Brackett, 1992). Others have reported a positive effect on maturation rates using media supplemented with vitamins (Naruse et al., 2007) or analogues of follicular-fluid meiosis-activating sterol (Grøndahl, 2008) or growth hormone (Menezo et al., 2006). In view of little available information on these nutrients at the time of study, they were not included in the culture media. Maturation rates significantly increased when supplemented culture media (FSH, HCG and oestradiol) were used in the present study. The rationale behind this (due to LH action of HCG) is based on the physiological role of FSH and HCG in oocyte maturation *in vivo*. FSH and LH (HCG) can directly or indirectly act on oocytes and cumulus cells to promote cytoplasmic maturation (Filali et al., 2008). It has been reported that human oocytes contain FSH and LH receptor mRNA that may play a role in oocyte maturation (Patsoula et al., 2003).

However, oocyte maturation rates do not reflect the spindle presence and aberrations. Therefore, a method for the quantitative and qualitative analysis of the spindle in the oocyte would be very helpful. FISH and immunofluorescence techniques were used to detect spindle abnormalities (Combelles et al., 2003; Makabe et al., 2001) but they are robust and require fixation of the oocyte and they therefore cannot be used clinically. Since it has been demonstrated that the mammalian oocyte is polarized the Polscope technology was employed to detect the spindle (Antczak and Van Blerkom, 1997; Edwards, 1965; Gardner, 1999; Inoué and Oldenbourg, 1998; Scott, 2000) due to its non-invasiveness (Wang et al., 2001a,b) safety and efficacy (Shen et al., 2008). The present study's results show that there was no significant difference in the presence of the spindle in oocytes matured under the two culture media as

Table 4 Chromosomal abnormalities (aneuploidy, polyploidy) observed in metaphase II oocytes and their corresponding polar bodies after in-vitro maturation. These results were obtained using double-labelling fluorescence in-situ hybridization for chromosomes 15 and 16.

	MII oocyte	*Polar body*
Number of MII oocytes and PB analysed	21	10
Aneuploidy	14 (66.7)	5 (50.0)
Polyploidy	2 (9.5)	2 (20.0)
Overall chromosome abnormalities	16 (76.2)	7 (70.0)
MII oocytes matching their corresponding polar body	7	—
Types of malsegregation found in metaphase preparations	Unbalanced separation of sister (homologous) chromatids Whole chromosome non-disjunction	
Whole chromosome non-disjunction (proportion of loss and gain%)	Chromosome loss: 1/3 (33.3%) for the oocyte and gain for the polar body Chromosome gain: 2/3 (66.7%) for the oocyte and loss for the polar body	

Values are number or number (%).
MII, metaphase II; PB, polar body.

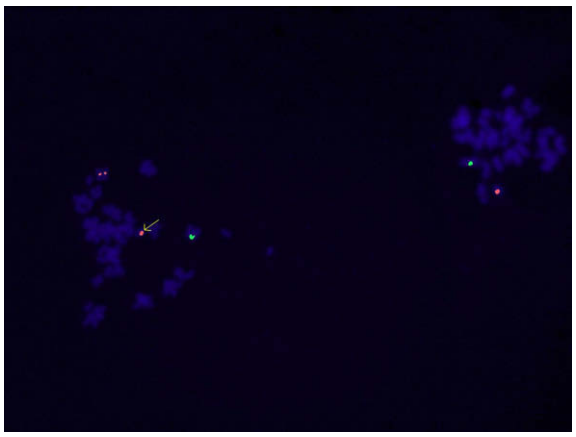

Figure 3 Epifluorescence microscopy image of a metaphase II oocyte, metaphase spread (left) and its corresponding polar body (right) after in-vitro maturation, obtained by using two-colour fluorescent in-situ hybridization. Chromosomes were counterstained with DAPI and a combination of two centromeric probes used for chromosome 15 (green) and chromosome 16 (orange). The extra chromatid for chromosome 16 on MII (arrow) (the polar body missing a chromatid for chromosome 16) represents an unbalanced chromatid separation (pre-division of sister chromatids) during anaphase I. Magnification 60×.

detected by the Polscope. In addition, the majority of oocytes with a birefringent spindle possessed normal chromosome alignment with organized microtubule structure after immunofluorescence analysis, which is in co-ordination with the results reported by Wang and Keefe (2002). However, this observation is not supported by an interesting study conducted by Coticchio et al. (2009) that reports a smaller percentage of fresh and frozen–thawed mature oocytes with normal spindle configuration after confocal analysis and a disconcordance between Polscope and immunofluorescence images, which argues against the predictive value of polarized microscopy for spindle dynamics. This discrepancy is even greater regarding their results of in-vitro matured oocytes which underlines that human spindles are easily destroyed during IVM or during oocyte meiotic maturation. Such a difference between the studies may depend on the maternal age of the participants, which is a dominant factor for oocyte quality (Battaglia et al., 1996). Other factors such as patient population, stimulation protocols, intravariance of spindle structure, sample size and time exposure to maturation media should also be taken into account. The possibility that variations may arise from the diversity of the two analyses cannot be excluded. In particular, the number of oocytes with normal spindles prevailed in studies employing conventional fluorescence methods rather than confocal microscopy (Coticchio et al., 2006). Nevertheless, by using a common observational analysis, there was no difference in the number of MII oocytes with abnormal spindle morphology between the two culture media. Spindle morphology may therefore mostly reflect oocyte handling *per se* rather than the culture media used in the maturation process, although little is known about the interactions of the oocyte with its environment.

This study also reports a high rate of in-vitro matured oocytes with their spindle closely aligned to the polar body in group A compared with group B. In other cases, the angular distance of the spindle to the PB of more than 5° has been correlated with cytoplasmic retention, maturation arrest (Moon et al., 2003) or developmental competency (Rienzi et al., 2003). Such a difference in these results is difficult to interpret since no discrepancy was evident in the percentage of abnormal spindle morphology between the two groups and it may reflect deficiency in the oocyte maturation process in the second group (group B) by unknown factors.

Spindle configuration is also crucial for high fidelity in chromosome segregation during meiotic divisions in the oocyte. Therefore disturbances in the spindle morphology may predispose oocytes to aneuploidy (Liu et al., 2000), which can reduce fertility and implantation rates (Eichenlaub-Ritter et al., 2002). Studies on chromosomal constitution of human oocytes, polar bodies and preimplantation embryos suggest that most errors occur at oogenesis (Kuliev et al., 2003; Munné, 2003; Verlinsky et al., 1996, 1998, 1999). Several processes such as whole chromosome non-disjunction, premature separation of sister (homologous) chromatids and gonadal mosaicism have been implicated in oocyte aneuploidy, (Pellestor et al., 2005). The commonest chromosomal abnormality in the current analysis was premature separation of sister chromatids and whole chromosome non-disjunction, which are also mentioned by larger cohort studies (Kuliev et al., 2003; Pellestor et al., 2002), and there was a 70% of concordance between the oocytes and their corresponding polar bodies. Although many groups have reported their experience with IVM, to date there is no reference to possible chromosome abnormalities of in-vitro matured oocytes (Edwards, 2007). A high incidence of aneuploidy was reported in embryos deriving from in-vitro matured oocytes in unstimulated (Requena et al., 2009) and stimulated cycles (Nogueira et al., 2000). The rate of chromosomal abnormalities of in-vitro matured oocytes and their corresponding polar bodies in this study is very high compared with the data reported when analysing both polar bodies of fresh oocytes for five (52%) (Kuliev et al., 2003) or for all of the chromosomes (67%) (Fragouli et al., 2009b). This discrepancy could arise from the fact that FISH analysis was restricted to in-vitro matured oocytes with abnormal spindle morphology. These unbelievably high rates of aneuploidy could also be attributed to culture conditions *in vitro* that may have permitted the oocytes to progress rapidly through maturation, which stresses key cellular pathways and leaves them predisposed to chromosome malsegregation (Eichenlaub-Ritter, 2000; Hassold and Hunt, 2001; Munné and Cohen, 1998; Munné et al., 1994; Plachot, 2003). It is also possible that abnormal oocytes are recognized and targeted for degeneration in the ovary, a control mechanism that might be disrupted by hormonal stimulation (Fragouli et al., 2009a) or by maturation conditions. Since the number of chromosomes analysed in this study is limited, the sample size was targeted and the artefactual loss of chromosomes/chromatids or FISH errors cannot be excluded, this study is viewed as pilot but it provides an impetus for examining further the chromosomal constitution of oocytes arising from IVM.

According to these data, supplemented media will give better results concerning the maturation rates but it does not necessarily warrant the spindle presence or its normal

morphology and chromosomal alignment. Accumulating evidence of the close relationship between spindle location, fertilization rates and embryonic competence suggest that, by using the Polscope, one could clearly benefit in the IVM process by providing a means of selecting viable oocytes for insemination in the IVF treatment. Since the spindle plays a crucial role during oocyte meiotic maturation (Hardarson et al., 2000; Wang and Keefe, 2002; Wang et al., 2001a,b), further studies using a larger number of samples are needed to relate spindle birefringence of in-vitro matured oocytes to aneuploidy and also assess, modify and improve culture media for IVM.

Acknowledgements

The authors wish to thank the clinicians, IVF embryologists and geneticists of 'Embryogenesis' for their co-operation in the development of the IVF/ICSI programme and the monitoring of ovarian stimulation cycles.

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Declaration: The authors report no financial or commercial conflicts of interest.

Received 29 January 2009; refereed 17 March 2009; accepted 22 December 2009.