Article

Meiotic spindle visualization in living human oocytes

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Abstract

A computer-assisted polarization microscopy system (polscope) has made it possible to analyse the meiotic spindle of oocytes subjected to intracytoplasmic sperm injection (ICSI) without affecting their viability. It has been shown that the presence of a detectable birefringent meiotic spindle inside the oocyte cytoplasm of human metaphase II (MII) prepared for ICSI is an indicator of oocyte quality, such as fertilization and developmental ability. Meiotic spindle imaging has also shown that this structure, when detectable, is not always aligned with the first polar body (PB1) in fresh MII oocytes. The relationship between the degree of meiotic spindle deviation from the PB1 location and ICSI outcomes is discussed in this paper. When the meiotic spindle of in-vitro matured oocytes is analysed, it is always found to be aligned with the PB1, suggesting that the misalignment observed in the oocytes matured *in vivo* results from the PB1 displacement during the manipulations for the cumulus and corona removal. Furthermore, polscope analysis of meiotic spindle changes in living MII oocytes subjected to freezing and thawing procedures has shown that the current techniques of oocyte cryopreservation cause meiotic spindle destruction. The polscope system may assist in the selection of fresh and thawed oocytes for ICSI.

Keywords: *cryopreservation, human oocyte, ICSI, meiotic spindle, polar body, polscope*

Introduction

At the stage of metaphase of the second meiotic division (MII), oocyte chromosomes are aligned at the equator of the meiotic spindle. This structure is highly dynamic, formed by microtubules located at the oocyte periphery with one pole attached to the cell cortex (Longo and Chen, 1985; Szollosi *et al*., 1986; Eichenlaub-Ritter *et al*., 2002, 2004). It appears that the location of the first polar body (PB1) is a poor indicator of the meiotic spindle position (Silva *et al*., 1999; Hardarson *et al*., 2000; Wang *et al*., 2001a,b,c). The angle deviation between the PB1 and the meiotic spindle is supposed to be due to movement of the PB1 rather than the meiotic spindle (Gardner, 1997).

The oocyte meiotic spindle is involved in various functions that are essential for fertilization and early post-fertilization events. First of all, it is responsible for proper chromosome segregation after oocyte activation. Moreover, a role for the oocyte meiotic spindle in establishing polarities and thus influencing polarity-sensitive events at fertilization has been suggested (Payne *et al*., 1993; Edwards and Beard, 1997), although, unlike some other animal species, sperm entry sites do not seem to be polarized in mammals (Santella *et al*., 1992; Edwards and Beard, 1997). Whether the point of sperm entry is related to the plane of the first cleavage remains a subject of controversy (Gardner and Davies, 2003; Johnson *et al*., 2003; Zernicka-Goetz *et al*., 2003). Finally, abnormal behaviour of the oocyte meiotic spindle can lead to the formation of multinucleated blastomeres with unusual ploidies resulting from uniparental inheritance (Van Blerkom *et al*., 2004).

The integrity of the meiotic spindle is necessary for the sequence of events leading to the correct completion of meiosis and fertilization. The microtubules of the meiotic spindle are, however, highly sensitive to chemical and physical changes that may occur during oocyte handling. It has been shown that oocyte cooling to room temperature dramatically affects the microtubular structure of the meiotic spindle, with deleterious consequences on chromosomal organization

(Sathananthan *et al*., 1988a; Pickering *et al*., 1990; Almeida and Bolton, 1995; Wang *et al*., 2001a; Zenzes *et al*., 2001). In addition, physiological parameters, such as increased maternal age (Battaglia *et al*., 1996; Volarik *et al*., 1998) and ageing *in vitro*, post-oocyte retrieval (Eichenlaub-Ritter *et al*., 2004), are associated with disruption of meiotic spindle architecture. The main and dramatic consequence of meiotic spindle damage is the formation of aneuploid embryos, by inducing unbalanced disjunction and non-disjunction of chromatids and chromosome scattering (reviewed by Bernard and Fuller, 1996). The meiotic spindle structure that controls chromosomal movement during meiosis is thus a key determinant of oocyte potential.

Because the meiotic spindle is not visible with conventional light microscopy (due to the lack of contrast), this structure has been imaged on fixed samples by immunofluorescence microscopy or electron microscopy (Moor and Crosby, 1985; Pickering and Johnson, 1987; Pickering *et al*., 1990; Aman and Parks, 1994; Almeida and Bolton, 1995). While these methods provide high-resolution images, they cause oocyte death, and thus have limited clinical use. The introduction of a novel orientation-independent polarized microscopy system (polscope) coupled with image processing software has allowed the visualization of meiotic spindle in a non-destructive way (Oldenbourg and Mei, 1995; Oldenbourg, 1999; Liu *et al*., 2000). The polscope system generates contrast to image the meiotic spindle on the basis of its birefringence. Parallel-aligned meiotic spindle microtubules are able to shift the plane of the polarized light, inducing retardation of the light. To enhance sensitivity, the system uses digital image processing such that meiotic spindle can be visualized irrespective of its orientation within the cell (Oldenbourg, 1999).

Because the position and the physical aspect of the meiotic spindle reflect its function, the ability to analyse the meiotic spindle in living oocyte with the polscope system has produced new information about oocyte quality and functionality and may help to reduce oocyte damage during handling and cryopreservation.

Laboratory procedure for meiotic spindle analysis

Intracytoplasmic sperm injection (ICSI) has offered the opportunity to study the meiotic spindle prior to in-vitro insemination, because for this technique the cells of the cumulus and corona radiata must be removed from the oocytes. Only denuded oocytes can be well visualized with the Polscope system for meiotic spindle evaluation.

The oocyte denudation is normally performed by brief exposure of the cumulus corona oocyte complex to HEPES-buffered medium containing hyaluronidase fraction VIII and by gentle aspiration in and out of a hand-drawn glass pipette. The denudated oocytes are then evaluated to assess their maturation stage. Mature (MII) and immature (MI) oocytes can be used for spindle observation.

For spindle observation and ICSI, the oocytes are placed in a microdrop of HEPES-buffered medium covered with mineral oil in a glass-bottomed culture dish. During handling and manipulation, extreme care must be taken to maintain the oocytes at 37°C. Because of the high sensitivity of the meiotic spindle to temperature variation, thermal stability of the oocytes is necessary to maintain meiotic spindle assembly. It has been shown (Wang *et al*., 2001a) that meiotic spindle are depolymerized even after slight fluctuations in temperature and thus become undetectable with the polscope system.

Meiotic spindle visualization is performed at ×20 and ×40 magnification, with LC polscope optics and controller (SpindleView; CRI, Cambridge, MA, USA), combined with a computerized image analysis system (SpindleView software, CRI). For this purpose, each oocyte is immobilized at the holding pipette, and rotated with the use of the injection pipette until both the meiotic spindle and the polar body are clearly in focus in the oocyte equatorial plane. Oocyte rotation around the axis connecting the centre of the oocyte with the PB1 allows orientation of the meiotic spindle microtubules, which often becomes more favourable for visualization of the structure's birefringence (Cooke *et al*., 2003; Rienzi *et al*., 2003). The procedure is very rapid (not more than 1 min per oocyte is necessary for the evaluation), easy and safe.

After imaging, ICSI procedure is normally performed.

Meiotic spindle analysis of fresh metaphase II oocytes

In order to assess the predictive value of meiotic spindle visualization for oocyte developmental ability, fresh MII oocytes have been evaluated by different authors prior to ICSI with the polscope system (Wang *et al*., 2001b,c; Cooke *et al*., 2003; Moon *et al*., 2003; Rienzi *et al*., 2003; Cohen *et al*., 2004). The percentage of oocytes displaying a detectable meiotic spindle varied between 60 and 90%. This difference seems to be related to two important parameters: (i) the thermal control during oocytes handling (Wang *et al*., 2001a, 2002); (ii) the technique of meiotic spindle visualization. Oocyte rotation during observation (Cooke *et al*., 2003; Rienzi *et al*., 2003) makes it possible to detect even meiotic spindle with relatively low brightness. With this manipulation, >90% of the oocytes were found to be positive for meiotic spindle evaluation in three different studies (Cooke *et al*., 2003; Rienzi *et al*., 2003, 2004).

Poor developmental ability after ICSI for oocytes with non-visualized meiotic spindles has been reported (Wang *et al*., 2001b,c; Cooke *et al*., 2003; Moon *et al*., 2003; Rienzi *et al*., 2003; Cohen *et al*., 2004) (**Table 1**). Meiotic spindles that can be visualized thus seems to be functionally superior to non-detectable meiotic spindles. Furthermore, embryos deriving from oocytes with poor functional meiotic spindles may have chromosomal abnormalities, which would explain the impaired cell development reported (Wang *et al*., 2001a,b,c, 2002).

The relationship between meiotic spindle location with regard to polar body position and ICSI results has also been recently addressed (Moon *et al*., 2003; Rienzi *et al*., 2003). In these studies, the position of the meiotic spindle was assessed at the time of ICSI, and ICSI results, in terms of the percentage of normally fertilized oocytes and of good quality embryos, were evaluated separately for groups of oocytes with different

degree of deviation of the PB1 from the meiotic spindle position.

The study from Moon and co-authors (2003) did not find any differences in fertilization rates and embryo quality among the different groups of oocytes analysed (**Table 2**).

Similarly, the results of a recent study (Rienzi *et al.*, 2003) have confirmed that there is no relationship between the displacement of the PB1 with regard to the meiotic spindle position on fertilization outcomes, but only when the angle of displacement does not exceed 90°. When ICSI was performed with oocytes whose polar body was displaced from the meiotic spindle position by >90°, lower rates of normal fertilization ensued (**Table 2**).

The difference found between the two studies might be explained by the low number of oocytes displaying high degrees of polar body displacement which could be analysed (22 and 24 oocytes for Moon *et al.*, 2003 and Rienzi *et al.*, 2003 respectively).

Meiotic spindle analysis of in-vitro matured oocytes

The polscope system can also be used on immature oocytes to assess the presence of the meiotic spindle during and after in-vitro maturation (**Figure 1**).

When oocytes that are found to be at metaphase I stage (MI) after cumulus oophorus and corona radiata removal are matured *in vitro* (for 3 h) and analysed with the polscope system, a birefrigent meiotic spindle can be detected in ~50% of the oocytes that had extruded the PB1 after in-vitro culture (Wang and Keefe 2002; Rienzi *et al.*, 2003) (**Figure 1c**).

Oocytes in which the meiotic spindle failed to be visualized may be immature, despite the presence of a clear PB1 in the perivitelline space. Interestingly, some oocytes were found to be at the stage of telophase I, with a clear meiotic spindle shared between the oocyte cytoplasm and the PB1 (**Figure 1b**). These oocytes would have been classified as 'mature' if analysed only with light microscopy. The use of the polscope system may thus also help to predict the true oocyte nuclear stage (Eichenlaub-Ritter *et al.*, 2002).

It has recently been shown that the meiotic spindle of in-vitro matured oocytes visualized with the polscope system is always aligned with the PB1 (Rienzi *et al.*, 2003). These observations differ from those obtained using immunostaining for tubulin in fixed in-vitro matured oocytes whose meiotic spindle was, on average, not located directly adjacent to the polar body, although the angle of deviation was significantly smaller than that measured in in-vivo matured oocytes (Hardarson *et al.*, 2000). A slight displacement of the meiotic spindle with regard to the polar body may thus result from oocyte fixation and processing for immunochemistry. The displacement of the meiotic spindle with regard to the PB1, observed in living human oocytes after enzymatic and mechanical treatment for cumulus–corona cell removal is thus likely to be a consequence of the corresponding manipulation. Because this manipulation is unlikely to influence intracellular organelle position, artefactual displacement of the PB1 from its original extrusion site is the most acceptable explanation. This observation is in agreement with other studies that have previously shown that, when the meiotic spindle is observed in human in-vivo matured oocytes that have not been exposed to any manipulation, its location always corresponds to the position of the PB1 (Baca and Zamboni, 1967; Dvorak and Tesarik, 1980). It is thus more pertinent to talk about PB1 displacement with regard to the meiotic spindle position than vice versa.

The studies conducted in this centre (Rienzi *et al.*, 2003) have also compared ICSI results between in-vitro matured oocytes with, and without, a detectable meiotic spindle (**Table 3**). Similarly to in-vivo matured oocytes, the absence of detectable meiotic spindles was associated with a lower incidence of normal fertilization and a lower proportion of good quality embryos as compared with oocytes in which the meiotic spindle was visualized (**Table 3**).

Meiotic spindle analysis during oocyte cryopreservation

The clinical efficacy of the current method used for human oocyte cryopreservation at the stage of MII is low. Alterations in the meiotic spindle structure are thought to be partly responsible for this situation (Sathananthan *et al.*, 1988a,b; Pickering *et al.*, 1990; Gook *et al.*, 1995).

The polscope system has gave the possibility to follow the behaviour of the meiotic spindle at different steps of the freezing and thawing procedure in the same human oocyte (Rienzi *et al.*, 2004). For this purpose, fresh MII oocytes have been cryopreserved in 1,2-propanediol with 0.1 mol/l sucrose using a slow freezing/rapid thawing programme and analysed step by step with the polscope system.

In all the oocytes that showed a meiotic spindle before freezing, the meiotic spindle remained detectable throughout the freezing procedure up to the step at which the oocytes were loaded into the cryopreservation straws. Immediately after thawing, the meiotic spindle was still visible in some oocytes (~35%), but it disappeared in all thawed oocytes in the subsequent washing steps involving cryoprotecting medium removal. The meiotic spindle, however, reappeared in all surviving oocytes after oocyte incubation at 37°C. All meiotic spindles present in cryopreserved MII oocytes are thus products of de-novo assembly in the post-thaw period. The fact that the spindle function in cryopreserved MII oocytes is entirely dependent on spindle reconstruction raises some concern about the functional capacity of these newly formed spindles.

The loss of the meiotic spindle during the thawing procedure has been confirmed by immunocytochemistry with anti-tubulin antibody (Rienzi *et al.*, 2004; Stachecki *et al.*, 2004). Nevertheless, no chromosomal dispersion has been observed after meiotic spindle depolymerization. The oocytes chromosomes seem to remain anchored and associated with the microtubule organizing centres even after meiotic spindle disassembly (Zenzes *et al.*, 2001). This observation is confirmed by the fact that the position of the reconstructed meiotic spindle is always the same as that of the original pre-freezing position in all cryopreserved oocytes analysed with the polscope system (Rienzi *et al.*, 2004).

Table 1. Relationship between the presence of a detectable meiotic spindle in fresh MII oocytes and the ICSI outcome (adapted from Wang *et al.*, 2001b; Moon *et al.*, 2003; Rienzi *et al.*, 2003).

	Presence of meiotic spindle in in-vivo matured MII oocytes					
	Yes			*No*		
	Wang *et al.* (2001b)	Moon *et al.* (2003)	Rienzi *et al.* (2003)	Wang *et al.* (2001b)	Moon *et al.* (2003)	Rienzi *et al.* (2003)
No. of injectedoocytes (%)	1266 (82.0)	523 (83.5)	484 (91.0)	278 (18.0)	103 (16.5)	48 (9.0)
No. of fertilized oocytes (%)	879 (69.4)[a]	430 (82.2)	362 (74.8)[b]	175 (62.9)[c]	79 (75.7)	16 (33.3)[d]
Good quality embryos per injected oocyte (%)	583 (46.0)[e]	276 (52.8)[f]	268 (55.4)[g]	97 (34.9)[h]	28 (27.2)[i]	9 (18.7)[l]

[a,c] $P < 0.05$.
[b,d] $P < 0.01$.
[e,h] $P < 0.01$.
[f,i] $P < 0.01$.
[g,l] $P < 0.01$.

Table 2. Relationship between first polar body deviation with regard to the meiotic spindle at the time of ICSI and fertilization and cleavage rates (adapted from Moon *et al.*, 2003; Rienzi *et al.*, 2003; only oocytes with detected meiotic spindle are evaluated).

	Deviations of polar body I from the meiotic spindle position							
	None		*Moderate*		*Medium*		*High*	
	Moon *et al.* (2003)	Rienzi *et al.* (2003)	Moon *et al.* (2003)	Rienzi *et al.* (2003)	Moon *et al.* (2003)	Rienzi *et al.* (2003)	Moon *et al.* (2003)	Rienzi *et al.* (2003)
No. of injected oocytes (%)	302 (57.7)	254 (52.5)	129 (24.7)	104 (21.5)	70 (13.4)	102 (21.1)	22 (4.2)	24 (4.9)
No of fertilized oocytes (%)	256 (84.8)	188 (74.0)[a]	105 (81.4)	78 (75.9)[b]	54 (77.1)	84 (82.3)[c]	17 (77.2)	12 (50.0)[d]
Good quality embryos per injected oocytes (%)	160 (53.0)	142 (55.9)	67 (51.9)	60 (57.7)	38 (70.6)	56 (54.9)	12 (54.5)	9 (37.5)

[a,d] $P < 0.01$.
[b,d] $P < 0.01$.
[c,d] $P < 0.01$.

Conclusions

Since the introduction of the polscope system a number of reports have shown a positive relationship between the presence of a detectable meiotic spindle and oocyte development ability after ICSI (Wang *et al.*, 2001b,c; Cooke *et al.*, 2003; Moon *et al.*, 2003; Rienzi *et al.*, 2003; Cohen *et al.*, 2004). The failure of meiotic spindle visualization in human oocytes might be due to temporary depolymerization of the microtubules, composing the structure subsequent, for example, to temperature variations (Wang *et al.*, 2001b). It has been shown that some of these oocytes can recover their meiotic spindles after being returned to the incubator, but the functionality of the recovered meiotic spindle seems to be impaired. Damaged meiotic spindles have been suggested to predispose oocytes to perturbation of chromosomal segregation, maturation arrest, and aneuploidy in the deriving embryos (Eichenlaub-Ritter *et al.*, 2002). Human oocytes found to be meiotic spindle-negative have, in fact, very low chances of achieving normal fertilization and embryo development, and of forming viable morulae or blastocysts (Wang *et al.*, 2001a,b; Cooke *et al.*, 2003; Moon *et al.*, 2003; Rienzi *et al.*, 2003). The absence of a detectable meiotic spindle or the presence of a detected but abnormal meiotic spindle may also reflect nuclear oocyte immaturity (oocytes that have failed to fully organize their meiotic spindle, despite the presence of the PB1 in the perivitelline space).

Little is known about optimal oocyte nuclear and cytoplasmic maturity before ICSI. The beneficial effect of a pre-incubation period prior to ICSI has been a matter of discussion in the literature. Some studies (Rienzi *et al.*, 1998; Ho *et al.*, 2003; Isiklar *et al.*, 2004) have shown that 2–3 h of oocyte pre-incubation may help to improve fertilization rates after ICSI. This observation was confirmed by a recent study (Cohen *et al.*, 2004) that has shown that more MII oocytes display a detectable meiotic spindle after at least 2 h of in-vitro culture. On the other hand, other studies failed to demonstrate a positive effect of oocyte pre-incubation on fertilization and embryo development rates of microinjected oocytes (Van de Velde *et al.*, 1998; Yanagida *et al.*, 1998; Jacobs *et al.*, 2001). It cannot be excluded that oocyte nuclear immaturity may not

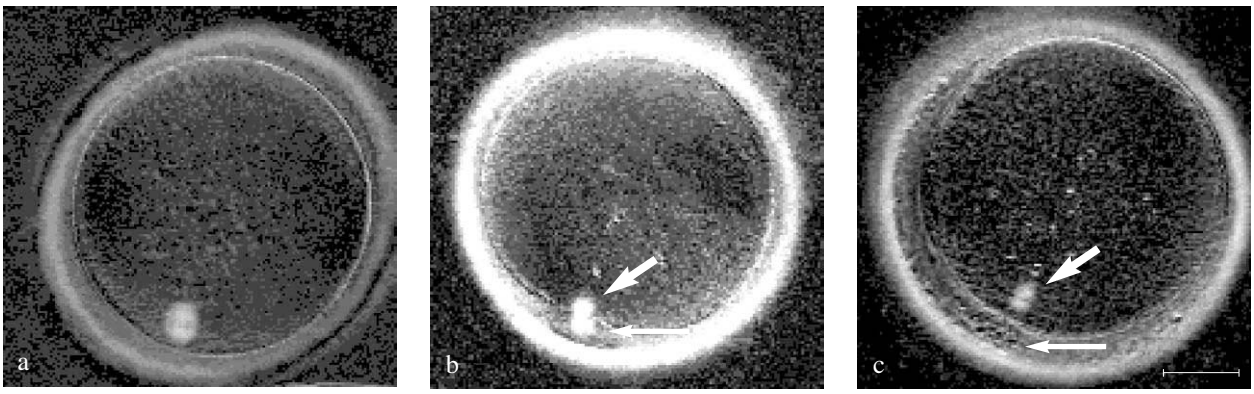

Figure 1. Sequential images of in-vitro maturation of a metaphase I human oocyte. The meiotic spindle (short arrow) is visible at the stage of metaphase I (**a**), telophase I (**b**) and metaphase II (**c**). The first polar body is already visible (long arrow) before complete oocyte nuclear maturation (panel b). Scale bar = 50 μm.

Table 3. Relationship between the presence of a detectable meiotic spindle in in-vitro matured MII oocytes and ICSI outcome (adapted from Rienzi *et al*., 2003).

	Presence of the meiotic spindle in in-vitro matured MII oocytes		
	Yes	*No*	*Total*
No. of injected oocytes (%)	28 (53.8)	24 (46.2)	52 (100)
No. of fertilized oocytes (%)	18 (64.3)[a]	8 (33.3)[b]	26 (50.0)
Good quality embryos per injected oocyte (%)	13 (46.4)[c]	3 (12.5)[d]	16 (30.8)

[a,b] $P < 0.05$.
[c,d] $P < 0.05$.

dramatically affect fertilization and early embryo development, but may have a deleterious effect later on during embryonic development. Because the polscope imaging does not impair oocyte viability (Keefe *et al*., 2003), it is believed that meiotic spindle analysis is a new and important indicator of oocyte maturity and quality which can help to clarify some important aspects of the ICSI procedure.

The orientation of the injection pipette during ICSI is usually chosen with regard to the position of the PB1, assuming that the meiotic spindle is located in a nearby region of the oocyte cytoplasm. However, the observations made with the polscope system have shown that the PB1 position does not predict accurately the location of the meiotic spindle in fresh MII oocytes (Wang *et al*. 2001b,c; Moon *et al*., 2003; Rienzi *et al*., 2003). The polar body has, in fact, been found to be mobile in the oocyte perivetelline space (Gardner *et al*., 1997).

The PB1 displacement with regard to the meiotic spindle position seems to be associated with abnormally strong mechanical deformation during the process of pipetting at the time of the corona radiata removal, which may also reflect oocyte cytoskeleton damage. Because meiotic spindles are highly sensitive to temperature variation, inappropriate oocyte handling would also cause irreversible damage to this structure, with dramatic consequences on oocyte developmental ability (Mandelbaum *et al*., 2004). The analysis of meiotic spindle presence and position with regard to the PB1 with the polscope system may thus help to improve oocyte manipulation and to minimize oocyte stress during handling.

In contrast to in-vivo matured oocytes, in-vitro matured oocytes have always the meiotic spindle in a location close to the PB1. In these oocytes, manipulation for the corona radiata removal occurs before polar body extrusion, which explains the spindle-to-polar body alignment in 100% of these oocytes. The percentage of in-vitro matured oocytes in which the meiotic spindle cannot be detected seems to be much higher as compared with in-vivo matured oocytes. This finding may explain the low rates of normal fertilization (De Vos *et al*., 1999) and cleavage (Cha and Chian, 1998), and the high rate of chromosomal abnormalities found in the subsequent embryos (Nogueira *et al*., 2000), when in-vitro matured oocytes are used for ICSI.

The study of the dynamic behaviour of the meiotic spindle in individual MII oocyte subjected to cryopreservation has shown that the current techniques of oocyte cryopreservation cause meiotic spindle destruction during oocyte thawing (Rienzi *et al*., 2004). The meiotic spindle can persist during prolonged incubation of the MII oocyte exposed to laboratory temperature while in the cryoprotecting medium, but inevitably disappears during the thawing process when the cryoprotecting medium is

washed from the oocyte. The meiotic spindles observed in thawed oocytes thus result from post-thaw reconstruction (Rienzi *et al*., 2004). The functional capacity of these recovered meiotic spindles has still to be determined. Encouraging data comes from a study performed on human embryos obtained with cryopreserved fresh MII oocytes, which have been analysed for chromosome status (Cobo *et al*., 2001). This study failed to find any significant differences in aneuploidy rate between embryos derived from cryopreserved oocytes as compared with those developing from fresh oocytes. Unfortunately, only a very low number of embryos have been analysed (21 and 51 embryos respectively). It is important to underline that cryopreservation seems also to have adverse consequences for the oocyte by inducing changes in mitochondrial ability to retain high polarity, and defects in Ca^{2+} signalling after insemination (Jones *et al*., 2004). A link between abnormal mitochondrial function in oocytes and disturbances in chromosome cohesion and segregation has been suggested (Eichenlaub-Ritter *et al*., 2004).

Oocyte selection that takes into account the presence and position of the meiotic spindle by using non-invasive examination with the polscope system seems to be very important, especially where for ethical, religious or legal reasons, the ICSI procedure must be performed with a minimum number of oocytes to avoid the destruction or cryopreservation of supernumerary embryos. The polscope system allows identification of immature oocytes or oocytes that have suffered for mechanical or temperature stress during handling. It is possible that in the near future, the polscope system will also be able to give information about structural order within the meiotic spindle that will serve as a new marker of oocyte quality.

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