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Spindle imaging: a new marker for optimal timing of ICSI?

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BACKGROUND: The LC Polscope facilitates visualization of the meiotic spindle in human oocyte. This study aimed to investigate meiotic spindle assembly in correlation to time elapsed after HCG administration, and to determine whether spindle imaging may serve to indicate the likelihood of fertilization and embryo cleavage. METHODS: Metaphase II (MII) oocytes from 103 couples who were being treated for male infertility were imaged with the Polscope prior to sperm injection. Spindle imaging was correlated to time elapsed from HCG administration, fertilization rate and embryo cleavage. The main outcome measures were spindle visualization, fertilization and embryo cleavage on day 3. RESULTS: A total of 770 MII oocytes were imaged. A spindle was imaged in a significantly higher number of oocytes from ⩾38 h after HCG administration compared with those in the <38 h group (78.1–81.5% versus 61.6%; $P < 0.001$). The fertilization rate in oocytes with a visible spindle was statistically higher compared with oocytes in which spindle could not be detected (70.4% versus 62.2%; $P = 0.035$). We found no relationship between spindle imaging and embryo cleavage on day 3. CONCLUSIONS: Spindle imaging, in addition to first polar body appearance, is an accurate indicator for oocyte maturity. We suggest that spindle imaging be performed prior to sperm injection.

Key words: HCG/ICSI/oocytes/Polscope/spindle

Introduction

Since its introduction more than a decade ago, ICSI has become a successful and well-established artificial reproductive technology (ART). The success of ICSI-embryo transfer partially depends upon oocyte nuclear and cytoplasmic maturation, as well as upon factors related to the methods of microinjection (Van de Velde *et al*., 1998). Currently, oocyte nuclear maturity is determined by the absence of germinal vesicle (GV) and the presence of an extruded polar body (PB). However, there is still uncertainty about optimal oocyte genetic and cytoplasmic maturity before sperm injection, and about whether there is any beneficial effect of a preinjection incubation period (Van de Velde *et al*., 1998; Yanagida *et al*., 1998; Jacobs *et al*., 2001).

Elevation of the estradiol level followed by the LH surge reinitiates meiosis in oocytes arrested in the dictyate stage of the first meiotic prophase. The changes in the activity of maturation promoting factor (MPF), c-mos kinases and mitogen-activated protein (MAP) kinases control the cell meiosis by promoting chromosome condensation, germinal vesicle break down (GVBD) and spindle formation, which occur in a stepwise manner (Eppig *et al*., 1994; Eichenlaub-Ritter and Peschke, 2002). This process ends in an oocyte at the metaphase of the second meiotic division, which becomes haploid after sperm penetration and release of the second PB (Eichenlaub-Ritter and Peschke, 2002).

Fluorescence *in situ* hybridization studies of preimplantation ICSI embryos indicate that the incidence of aneuploidy and other chromosomal abnormalities may be >50% (Munne *et al*., 1998). Aneuploidy (trisomy or monosomy) is the most commonly identified chromosome abnormality in humans and the principal genetic cause of pregnancy loss, with most aneuploid conceptuses perishing in the uterus (Hassold and Hunt, 2001). Relatively little is known of the mechanisms by which trisomy and monosomy originate in humans, yet the incidence of aneuploidy in natural pregnancies is reported to be at least 5%, and possibly as high as a staggering 25% (Hassold and Hunt, 2001). This emphasizes clearly the complexity of meiotic division and its susceptibility to errors (Champion and Hawley, 2002).

Errors of chromosomes segregation in the oocyte are more common during the first meiotic division. Possible underlying

mechanisms are: failure to resolve chiasmata between homologous chromosomes at anaphase I, disturbances in the recombination pathway and premature separation of sister chromatids. Metaphase II (MII) errors are thought to result from the failure of sister chromatid separation (Hassold and Hunt, 2001). The meiotic spindle controls chromosomal movement through the different stages of meiosis, thus playing a central role in the successful completion of meiosis. It has been suggested that defects in meiotic spindle formation can be associated with non-disjunction, which results in aneuploid gametes and aneuploid embryos (Pickering *et al*., 1988; Battaglia *et al*., 1996; Champion and Hawley, 2002; Eichenlaub-Ritter *et al*., 2002).

In order to prevent the development of aneuploid embryos, we are constantly searching for oocyte quality parameters that will predict the success of the ICSI cycle and future embryo traits. Cumulus–oocyte complex and oocyte morphology, however, are poor markers of oocyte quality (De Sutter *et al*., 1996; Rattanachaiyanont *et al*., 1999).

The recent introduction of a new type of polarized light microscope facilitates a non-invasive visualization of the meiotic spindle in the live human oocyte (Wang *et al*., 2001a; b; c), thereby enabling a better assessment of oocyte meiotic stage. Spindle imaging raises new questions: should an oocyte that is without a GV and with an extruded first PB, and which is considered to be arrested in the second metaphase, have aligned chromosomes in the metaphase plate? When can one expect to observe chromosomal alignment in the metaphase plate?

Lack of efficient checkpoints in the oocyte during maturation permit fertilization of oocytes with chromosomal aberrations (Champion and Hawley, 2002). It is hoped that spindle imaging can potentially identify oocytes at high risk for non-disjunction, and thus avoid injection of oocytes that have failed to fully organize their meiotic spindle and have not reached full meiotic maturity. Wang *et al*. (2001b) recently reported that spindle imaging with the Polscope (CRI, Cambridge, MA, USA) could predict embryo developmental competence and fertilization rate by better preinjection assessment of oocyte quality.

The current study was conducted to investigate meiotic spindle assembly in association with the time that had elapsed after HCG administration, and to determine whether spindle imaging may serve to indicate the likelihood of fertilization, as well as serve as a marker to predict embryo cleavage.

Materials and methods

Patients

The study group included 103 couples (average age 33 ± 5.6 years, range 22–43) who were being treated in our IVF unit for male factor infertility. Controlled ovarian stimulation was performed by means of one of our four routine protocols: GnRH analogue (Buserelin acetate nasal spray; Hoechst AG, Frankfurt, Germany)/HMG (Pergonal; Teva Pharmaceutical Industries Ltd, Petah Tikva, Israel), 58 cycles; GnRH analogue/recombinant FSH (rFSH) (Gonal-F; Serono, Aubonne, Switzerland), 28 cycles; GnRH antagonist (Cetrotide; Serono International SR, Geneva, Switzerland)/HMG, seven cycles; and GnRH antagonist/rFSH, 10 cycles.

When the follicles reached a mean diameter of 17 mm, 10 000 IU HCG (Chorigon; Teva Pharmaceutical Industries Ltd) was administered. Oocyte retrieval by an ultrasound-guided transvaginal approach was scheduled 35–36 h later.

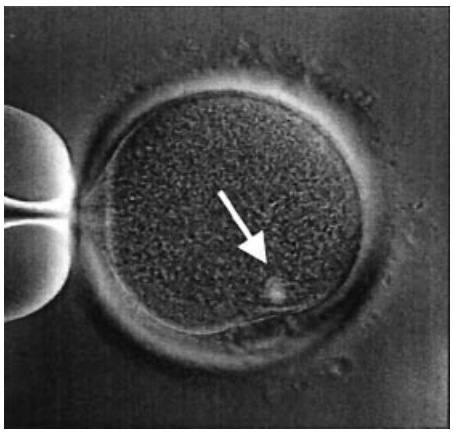

Figure 1. The meiotic spindle imaged by the Polscope (arrow).

Micromanipulation and spindle imaging

The cumulus–oocyte complexes were cultured in a P1 medium (Irvine Scientific, Santa Ana, CA, USA) supplemented with 16% synthesized serum substitute (SSS) (Irvine Scientific) after aspiration. Cumulus cells were removed with hyaluronidase (MediCult, Copenhagen, Denmark; 80 IU/ml) in flushing medium (Irvine Scientific). Denuded oocytes were assessed for their meiotic stage according to the presence or absence of GV and first PB. Only MII oocytes (without GV and with a first PB) were washed and transferred to mHTF (Irvine Scientific) supplemented with 5% SSS in glass Petri dishes for spindle observation.

Oocytes were imaged in a Nikon light microscope equipped with an LC Polscope (CRI Instruments) controller and CCD camera. Images were evaluated using the LC Polscope imaging software. The spindles were imaged at ×400 magnification (Figure 1). Those oocytes in which the spindle was not observed were rotated to three different positions before being marked as lacking a spindle. The oocytes were imaged only once, and rigorous temperature control was maintained in order to minimize environmental changes outside the incubator. The time that had elapsed since HCG administration was recorded, and was correlated to the findings on spindle imaging. In most cases, the sperm injection procedure was carried out immediately after spindle imaging. The isolated sperm was injected into the oocyte, taking special care not to damage the spindle structure. After the ICSI procedure, injected oocytes were returned to the culture dish for further incubation. Oocytes identified as having a spindle were separated from those in which no spindle could be observed.

Assessment of fertilization and embryo cleavage

Oocytes were inspected under a stereomicroscope (Olympus, SZH, Tokyo, Japan) for fertilization (2PN) 18–20 h after sperm injection. Embryo cleavage was evaluated 72 h after ICSI, prior to embryo transfer. The embryos were classified into three groups: (i) low cleavage rate, <5 cells; (ii) moderate cleavage rate, 5–7 cells; and (iii) good cleavage rate, ⩾8 cells. Because of weekend considerations, in a small proportion of the patients embryos were transferred or cyopreserved on day 2, without being morphologically assessed.

Time intervals between HCG administration and spindle detection, and relationships between an existing spindle and fertilization and embryo cleavage rate

We searched for differences in spindle imaging after three different time intervals from HCG administration: group 1 was imaged 36–37 h after HCG administration; group 2 was imaged after 38–39 h; and group 3 was imaged after 40–42 h. These differences in time intervals were the result of our laboratory's processing schedules. We also searched for an association between the presence of a spindle and fertilization and the embryo cleavage rate on day 3.

Statistical analysis

A logistic regression test was used to compare spindle imaging in the three groups according to different time intervals from HCG administration to spindle imaging. Two-way analysis of variance and χ^2-tests were used to compare embryo cleavage and fertilization rates. The values are presented as mean ± SD. $P < 0.05$ was considered statistically significant.

Results

This study included 770 oocytes, from 103 ICSI cycles, which extruded the first PB. The spindle could be imaged in 585 oocytes (76%) and was not imaged in 185 oocytes (24%).

Effect of the time elapsed from HCG administration to spindle imaging

In order to examine the effect of time that had elapsed from HCG administration to spindle imaging, the oocytes were retrospectively divided into three groups (Table I). Spindle imaging in groups 1, 2 and 3 was possible in 61.6, 81.5 and 78.1% of oocytes, respectively. In a logistic regression test, spindle imaging in groups 2 and 3 was found to be significantly higher ($P < 0.001$) compared with group 1.

The percentage of oocytes in which the spindle was not imaged was similar for the four ovarian stimulation protocols. Furthermore, the oocytes from the different stimulation protocols were equally distributed according to their relative size in the three groups (Table II).

Relationship between spindle imaging, fertilization rate and embryo cleavage on day 3

The fertilization rate in oocytes with a visible spindle, pooled together, was significantly higher compared with oocytes in which spindle could not be detected (70.4% versus 62.2%; $P = 0.035$) (Table III).

Analysis of the fertilization rate separately for each time group is presented in Table IV. The fertilization rate of spindle (+) oocytes compared with spindle (–) oocytes in group 2 was of borderline significance ($P = 0.065$). Furthermore, spindle (+)

Table I. Spindle imaging according to time elapsed from HCG administration

	Group 1 36–37 h	Group 2 38–39 h	Group 3 40–42 h
Number of oocytes imaged	177	378	215
Oocytes with spindle (%)	109 (61.6)	308 (81.5)[a]	168 (78.1)[a]

[a]Spindle imaging in groups 2 and 3 was found to be significantly higher ($P < 0.001$), compared with group 1.

Table II. Ovarian stimulation protocols and distribution of oocytes in the three time groups

Ovarian stimulation protocol	Pooled no. of oocytes	No. of oocytes without spindle (%)	No. of the included oocytes from the total number[a] (%)		
			Group 1	Group 2	Group 3
GnRH analogue/HMG	418	98 (23.4)	97/177 (54.8)	198/378 (52.4)	123/215 (57.2)
GnRH analogue/rFSH	209	54 (25.8)	52/177 (29.4)	100/378 (26.5)	57/215 (26.5)
GnRH antagonist/HMG	45	9 (20)	7/177 (4)	16/378 (4.2)	22/215 (10.2)
GnRH antagonist/rFSH	98	24 (24.5)	22/177 (12.4)	63/378 (16.7)	13/215 (6)

[a]The numerator refers to the included oocytes, which was derived from the particular stimulation protocol, and the denominator refers to the number of imaged oocytes, as presented in Table I.

Table III. Pooled fertilization rate and embryo cleavage

Spindle imaging	No. of oocytes (%)	No. of fertilized oocytes (%)[a]	No. of embryos assessed on day 3[b]	Embryo cleavage rate on day 3 (%)[c]		
				<5 cells	5–7 cells	>8 cells
Spindle (+)	585 (76.0)	412 (70.4)	358	92 (25.7)	97 (27.1)	169 (47.2)
Spindle (–)	185 (24.0)	115 (62.2)	90	30 (33.3)	28 (31.1)	32 (35.6)

[a]Fertilization rate was found to be significantly higher in oocytes with a visible spindle compared with oocytes in which no spindle was demonstrated ($P = 0.035$).
[b]The decline between 2PN embryos and the number of embryos assessed on day 3 is due to day 2 embryo transfer or cryopreservation.
[c]Day 3 embryo cleavage rate was not statistically different in oocytes with a visible spindle compared with oocytes in which no spindle was demonstrated.

Table IV. Fertilization rate and embryo cleavage with subdivision into three time intervals

	36–37 h		38–39 h		40–42 h	
	Spindle +	Spindle –	Spindle +	Spindle –	Spindle +	Spindle –
No. of injected (MII) oocytes	109	68	308	70	168	47
No. of 2PN embryos and fertilization rate (%)	73 (67)	41 (60.3)	222 (72.1)	42 (60)	117 (69.6)	32 (68.1)
No. of embryos assessed on day 3	66	36	186	30	106	24
Day 3 cleavage rate (%)						
<5 cells	18 (27.3)	16 (44.4)	42 (22.6)	9 (30)	32 (30.2)	5 (20.8)
5–7 cells	17 (25.8)	8 (22.2)	52 (28)	12 (40)	28 (26.4)	8 (33.3)
>8 cells	31 (47)	12 (33.3)	92 (49.4)	9 (30)	46 (43.4)	11 (45.8)

oocytes in groups 1 and 3 achieved higher, albeit not statistically significant, fertilization rates compared with spindle (–) oocytes.

The embryo cleavage rate on day 3 was not statistically different between oocytes with and without spindles (Table III). The subdivision into time groups (Table IV) resulted in small groups, which further weakened the statistical power.

Discussion

As part of the ongoing search for markers that predict higher embryo developmental competence and higher fertilization rate by better preinjection assessment of oocyte maturation and quality, it was suggested that spindle imaging with the new Polscope technology could contribute valuable information (Wang *et al*., 2001b). In the present study, we found that the spindle was not imaged in 24% (185/770) of oocytes with a first PB. These findings are comparable to previous studies by Wang *et al*. (2001a, b) and Moon *et al*. (2003), whose comparable figures were 18, 38.6 and 16.5% of oocytes, respectively. Furthermore, when we compared oocytes at different time intervals from HCG administration, we found that there was a significantly lower number of oocytes in which the spindle was imaged at 36–37 h from HCG (61.6%, 109/177), compared with 38–39 h (81.5%, 308/378) and 40–42 h (78.1%, 168/215) from HCG ($P < 0.001$).

The fertilization rate for oocytes in which the spindle was imaged by the Polscope (70.4%, 412/585) was superior to that of oocytes in which no spindle was imaged (62.2%, 115/185) ($P < 0.05$) (Table III). These findings are in accordance with previous reports by Wang *et al*. (2001a, b); however, Moon *et al*. (2003) recently reported that the fertilization rate was not significantly different. Analysis of the fertilization rate separately for each time group disclosed a borderline significance in group 2 only. Furthermore, the majority of oocytes pooled in were derived from experimental group 2. We did, however, find an impact of spindle presence on the fertilization rate in each individual subgroup. These findings stem from the fact that analysing the subgroups means there are fewer numbers in each subgroup, and subsequently there is a decease in statistical power. Since our time groups were retrospectively analysed and therefore were not equal in size, clarification of this issue awaits future prospective studies.

We found no correlation between spindle imaging and embryo cleavage on day 3. These results are contrary those of Wang *et al*. (2001b), who reported a significant difference to be detected on day 3 and a highly significant difference on days 5 and 6, and Moon *et al*. (2003), who reported a significant difference on day 3.

It is worth noting that the number of embryos that were followed up to day 3 of development derived from oocytes without a spindle was 48.6%, compared with 61% in oocytes with spindle; this is mainly related to the fertilization rate. However, a further decline is noted between the number of the 2PN embryos and the number of embryos assessed on day 3. This decline is mainly attributed to day 2 transfers and cryopreservation, which were performed in a small proportion of the patients (because of weekend considerations), rather than selection of 2PN embryos.

Nonetheless, all these recent findings raise the question about the significance of spindle imaging and how can it contribute to our assessment of oocyte quality. Spindle assembly is affected by several factors, such as the microtuble motor proteins dynein and dynactin. MPF, c-mos kinases and MAP kinases are mediators of spindle formation and cell cycle (Eichenlaub-Ritter and Peschke, 2002); thus, it is hypothesized that oocytes lacking spindles do not reach full maturity as a result of disturbance in the signalling pathways, lack of protein complexes that support spindle formation or low energy supply (Eichenlaub-Ritter and Peschke, 2002; Eichenlaub-Ritter *et al*., 2002). Another possibility is that spindle is disassembled because of fluctuations in temperature (Pickering *et al*., 1990; Wang *et al*., 2001b, c). In order to eliminate this latter possibility we employed rigorous thermal control during oocyte imaging. It is still possible, however, that oocytes in which spindle was not imaged are within the normal range, and that there is no clinical significance.

The findings of the present study clearly demonstrate that the spindle is imaged in a significantly higher number of oocytes (78.1–81.5%) $\geqslant$38 h after HCG compared with the <38 h group (61.6%). To the best of our knowledge, this is the first attempt to correlate spindle imaging in a large group of live human oocytes to the time that had elapsed from HCG administration. Our findings support the observations made on oocytes from natural and IVF cycles in which chromosome alignment on the second metaphase plate was demonstrated within 44 h after HCG (Battaglia *et al*., 1996). According to our results and those of Wang *et al*. (2001a, b), higher rates of fertilization are achieved among oocytes in which a spindle was demonstrated; thus, an early injection (before 38 h have elapsed since HCG)

increases the likelihood of injecting an oocyte lacking a spindle, and this may have a deleterious effect on the chances of fertilization.

There is still uncertainty with regard to the importance of oocyte maturity before its injection and to the beneficial affect of a preinjection incubation period. Although Rienzi *et al*. (1998) found a lower fertilization rate in oocytes injected before 39 h had elapsed since HCG administration, the majority of studies evaluating different timing of sperm injection vis-à-vis fertilization rates and embryo development did not demonstrate any effect of early (37–38 h after HCG) and late (41–42 h after HCG) oocyte injection on fertilization rate (Van de Velde *et al*., 1998). Furthermore, Yanagida *et al*. (1998) and Jacobs *et al*. (2001) did not find any statistically significant differences when oocytes were incubated for between 1 and 9 h and between 0.5 and 8 h, respectively. In the present study, however, we examined the effect of spindle imaging rather than time elapsed from HCG *per se*.

We scanned the oocytes only once. There is no question that serial scanning over time is a better way to evaluate oocyte meiotic maturation; however, we choose to minimize the oocyte's exposure outside the incubator and used only one scan. Although our results are based on a single scan, they show that a spindle is imaged in a significantly higher number of oocytes from ⩾38 h after HCG administration and, for the first time, it appears that we have a better marker for the oocyte's meiotic stage than the presence of the first PB alone. Since the ICSI procedure overrides the natural synchronization of sperm penetration into the oocyte, future research should focus on the consequences and safety of injecting an oocyte in which no spindle could be demonstrated.

Our working hypothesis was that oocytes with an altered spindle assembly are at higher risk for chromosomal abnormalities that are likely to result in aneuploid gametes with subsequent aneuploid embryos. Altered spindle assembly can be the result of aberrant signalling pathways during oocyte growth or low energy supply, resulting in both cytoplasmic and nuclear immaturity that will further compromise fertilization and embryo development (Eichenlaub-Ritter and Peschke, 2002; Eichenlaub-Ritter *et al*., 2002). The lower fertilization rate of oocytes in which a spindle was not imaged can be attributed to either nuclear and/or cytoplasmic immaturity; however, 62.2% of oocytes lacking a spindle are still fertilized. Possible explanations for this are that a spindle was assembled in the short period of time between imaging and injection, or after it, or that the lack of a spindle does not prevent fertilization in all cases, but it is possible that it will have an affect later on during embryonic development. In the meantime, whether the absence of spindle imaging represents nuclear immaturity remains an open question. Although we did not find a correlation between spindle imaging and embryo cleavage rate on day 3, a significant difference on day 3 and a highly significant difference on days 5 and 6 has been reported previously (Wang *et al*., 2001b). As also mentioned above, Moon *et al*. (2003) found a significant difference on day 3. Our different embryo cleavage classification is one possible explanation for the difference in these findings. Embryonic genomic activation, however, is expected at the 4–8 cells stage (Artley *et al*., 1992; Munne *et al*., 1994), which coincides with spontaneous embryonic cleavage arrest. Since our study's endpoint was at day 3, it might be too early for chromosomal aberrations to impact embryo cleavage, and may also explain the highly significant difference between embryo cleavage on day 3 and on days 5 and 6 that was reported by Wang *et al*. (2001b).

In conclusion, the current criteria for an MII oocyte, the absence of GV and an extruded first PB, might be inadequate for a comprehensive assessment of meiotic maturation. The meiotic spindle, imaged by the Polscope for the determination of oocyte maturity, appears to be a better indicator than the presence of the first PB alone. Furthermore, these results also indicate that the presence or absence of a spindle constitutes an additional parameter for predicting fertilization. More oocytes display the meiotic spindle of the second metaphase arrest starting at 38 h after HCG administration, and thereafter. Further prospective studies are needed to support these findings and to assess the pregnancy outcome, in order to determine whether postponement of ICSI until 38–42 h after HCG administration should be applied.

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