—Mini Review—

ICSI with the assistance of meiotic spindle imaging for the production of high quality embryos

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Abstract: To avoid multiple pregnancies, it is important to select a single high quality embryo for transfer. Observation of meiotic spindles in oocytes is one way of evaluating embryo developmental competence. The spindle is non-invasively visualized by polarized light microscopy. It is possible to perform ICSI more safely and with optimal timing during meiosis by visualizing the meiotic spindle just before ICSI. In some human studies, it has been reported that oocytes with a spindle show significantly higher fertilization, blastocyst formation and pregnancy rates. Recently, it has been reported that morphological spindle normality is related to the live birth rate. In mice, the areas of the spindles in aged oocytes (23 h after hCG injection) are larger than those in young oocytes (15 h after hCG injection), and the blastocyst formation rate of aged oocytes is lower than that of young oocytes. Addition of MG132, a ubiquitin-mediated proteasome proteolysis inhibitor, to the medium prevents the spindle from becoming large and increases the blastocyst developmental rate. Therefore, it is thought that the spindle configuration and chromosome arrangement reflect the physiological stage or quality of an oocyte. Spindle imaging provides a useful index for comprehensively assessing embryo quality.

Key words: Spindle imaging, PolScope, ICSI, Human, Aging

Introduction

In 1992, a healthy baby was born after replacing a single embryo, which was fertilized by intracytoplasmic sperm injection (ICSI) [1]. This was the first successful human pregnancy and live birth by ICSI [2, 3]. Since then, the use of ICSI has rapidly spread worldwide, and it has become a standard treatment for infertility due to severe semen abnormalities in human assisted reproduction technology (ART). Today, indications for ICSI are not male infertility alone; failure of fertilization in IVF cycles is also considered suitable for ICSI treatment [4].

Along with the progress of ART, multiple pregnancies have gradually increased. To increase the pregnancy rate after embryo transfer, multiple embryos were often transferred at one time. A major complication of ART treatment is recognized as multiple pregnancies [5–7]. Elective transfer of a single embryo (eSET) is an effective way of reducing the risk of multiple pregnancies. At the point of execution, the key to the success of eSET is the developmental competence of the single embryo. Therefore, the selection of a good quality embryo is crucial. Many studies have assessed oocyte or embryo viability, and their criteria for selection, e.g. oxygen consumption, metabolic profiling and gene expression [8–11]. In clinical practice, recent advances in oocyte or embryo assessment are: the evaluation of meiotic spindles in human oocytes by polarized light microscopy [12, 13], and the morphokinetics of embryos by time-lapse monitoring [14, 15].

In ICSI, cumulus cells are removed by hyaluronidase and pipetting, and the oocytes are fertilized after morphological evaluation. With cumulus-free oocytes it is easy to make detailed observations of the ooplasma and the maturity of the oocytes. In addition, non-invasive observation of the meiotic spindle may provide more information on oocyte quality. However, the relationship between the meiotic spindle image and the competence of embryo development is not clear, and is not well understood. Recently, the age of ICSI patients has been gradually increasing, and the quality of oocytes decreases, and the miscarriage rate increases dramatically with increasing age [16]. Against this background, we think that the evaluation of oocyte quality is becoming very im-

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portant in ART.

The objective of this review is to summarize meiotic spindle evaluation in the human oocyte by polarized light microscopy, and the clinical use of spindle images to assess the embryo quality. Furthermore, we share our experience and recent findings in the clinical use of polarized light microscopy.

Non-invasive Observation of Oocyte Spindles

Spindles are composed of microtubules that show birefringence, which cytoplasm does not. Polarized light microscopy has the potential to visualize and measure birefringent structures such as spindles. At present, three types of polarized light microscope system are available for the observation of spindles in oocytes. Photographs of human oocytes by bright field microscopy, the OCTAX polar AIDE system (MTG), the IX-Robopolar system (Olympus), and the Oosight imaging system (PolScope, CRi) are shown in Figs. 1A, B, C, and D, respectively. Observation of the spindle does not damage an oocyte, nor does it affect the developmental competence of an oocyte. Thus, it allows safer fertilization of oocytes by ICSI in clinical settings.

Spindle observation has two main merits. The first is the detection of the spindle location during ICSI. The visualization of the metaphase II (MII) spindle in oocytes by polarized light microscopy can be used to avoid damaging the spindle and chromosomes when inserting the ICSI needle into the ooplasma. In the basic technique of human ICSI, the injection is usually made at the 3 or 9 o'clock position with the first polar body (PB) at the 12 or 6 o'clock position, because it is postulated that the MII spindle is located adjacent to the first PB. Recent observations of the relationship between the MII spindle and the first PB by fluorescent immunostaining and polarized light microscopy show that the MII spindle is not always present near the first PB at ICSI [17–19]. The position of the MII spindle cannot be predicted by the location of the first PB. Uchiyama *et al.* [20] reported that when the spindle was intentionally damaged by cytoplasm aspiration, the normal fertilization rate decreased and the multipronuclear formation rate increased. Therefore, for safe ICSI, it is thought that the injection should be made at the 3 or 9 o'clock position with the MII spindle visualized using a polarized light microscope at the 12 or 6 o'clock position to avoid damaging the spindle.

The second merit of spindle observation is that the spindle image provides an indication of the optimal timing of ICSI. Time-lapse observation of MI to MII oocytes has shown that the presence of the first PB does not necessarily indicate completion of spindle disassembly [21, 22]. After extrusion of the first PB, the spindle still maintains a connective strand between the first PB and the ooplasm for approximately 75 and 90 min at telophase I. Furthermore, for 40 to 60 min, the spindle is not detected. After approximately 115 to 150 min after the first PB extrusion, the MII meiotic spindle is formed, and can be observed by polarized light microscopy [21, 23]. Montag *et al.* [24] reported that when oocytes at telophase I were fertilized by ICSI, the majority of oocytes showed three pronuclei and a missing second PB 16–20 h after ICSI. Also, in oocytes with the first PB which did not have a MII spindle, it became visible in half of the oocytes after additional culture for 2 h, and the fertilization rate was similar to that of oocytes in which the spindle was detected at the first observation [21].

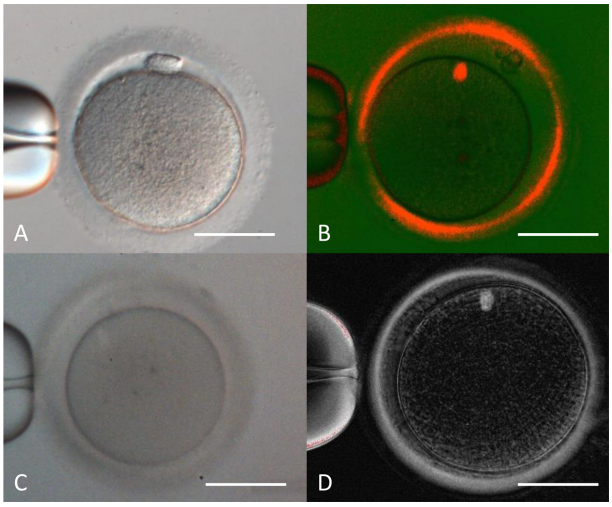

Fig. 1. A human oocyte observed by bright field light microscopy (A), and polarized light microscopy: OCTAX polar AIDE system (B), IX-Robopolar system (C), and Oosight imaging system (D). A spindle in a MII oocyte becomes visible under polarized light microscopy. Scale bar is 50 μm.

Several studies have investigated the importance of the presence of a spindle in human oocytes, but their results are contradictory. Some studies have reported higher fertilization rates in oocytes with spindles at the time of ICSI [25–31], whereas other studies have found no significant difference in fertilization rates [32, 33]. However, a recent meta-analysis found that oocytes with a visible spindle exhibited statistically higher fertilization, cleavage and blastocyst rates [34]. In our clinic, we have confirmed that oocytes with a visible spindle have a significantly higher fertilization rate (82.8% vs. 39.4%) and blastocyst rate (43.4% vs. 10.3%) than oocytes with

Table 1. Fertilization and blastocyst rates of human oocytes with or without a visible spindle after ICSI

Parameter	Visible spindle	Not-visible spindle	Significance *P*
No. of patients	1655	129	-
Maternal age	40.6 ± 0.1	40.0 ± 0.4	NS
No. of oocytes	3455	155	-
Fertilization (2PN) (%)*	2861 (82.8)	61 (39.4)	$P < 0.0001$
Blastocysts (%)**	1501 (43.4)	16 (10.3)	$P < 0.0001$

*Normal fertilization rate per oocytes. **Blastocyst rate per oocytes. Oocytes were collected in letrozole-stimulated cycles. The *t*-test was used to compare maternal ages. Data are expressed as the mean ± SD. Fisher's exact test was used to compare fertilization and blastocyst rates.

no visible spindle (Table 1). These results show that we should not estimate the oocyte maturity by the presence of the first PB alone. Rather, observation of the MII spindle in oocytes by polarized light microscopy gives us more information on oocyte quality and provides the best estimate of the optimal timing for ICSI. The estimation of the best timing for ICSI is the first step toward increase of normal fertilization and cleavage rates, and consequent improvements to the outcome of treatment in human ART.

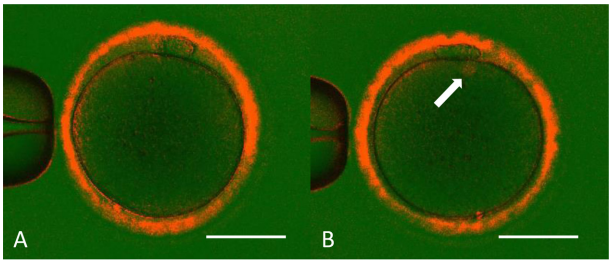

Fig. 2. At the first observation, a spindle was not detected in a human oocyte (A), but after additional culture for 1 h, a spindle (white arrow) appeared under the first polar body (B). Scale bar is 50 μm.

Use of Spindle Imaging to Select High Quality Oocytes

The meiotic spindle plays a key role in accurate chromosome segregation and chromosomal movement during meiosis. Whether or not a spindle image by PolScope (CRi) is a marker of oocyte quality has previously been discussed. The normal fertilization rate of oocytes with a spindle at the time of ICSI has been reported to be higher than that of oocytes without spindles [25–31]. However Moon *et al.* [32] reported that the normal fertilization rate showed no significant difference between oocytes with and without spindles. Furthermore, embryo grade at Day 2 or 3 in a group of oocytes with spindles was similar to that of a group of oocytes without spindles [17, 18].

Madaschi *et al.* [31] reported that the majority of oocytes with visible spindles after ICSI cleaved early within 27 h after ICSI, and the pregnancy rate of those embryos was higher than that of oocytes without a spindle. When spindles in human oocytes matured for 22 and 24 h *in vitro* could not be detected by PolScope, abnormal microtubule organization and abnormal chromosome alignment were frequently observed in the oocytes under confocal microscopy after fluorescence immunostaining [35].

During normal meiotic maturation, spindles in oocytes are not always observed. However, it is not always the case that oocytes without spindle images have abnormal chromosomes [21]. It has been reported that the visible spindle rate in oocytes at 38 h post-hCG is higher than at other times [27]. Oocytes without spindle images may also complete meiotic maturation. Thus, we think that the time of observation is an important factor in the evaluation of oocyte quality by spindle imaging.

At our clinic, when meiotic spindles in the oocytes are not detected at the first observation, the oocytes are cultured for a further 1 to 3 h depending on the situation. This short culture may result in spindle visualization (Fig. 2A and B), which increases the fertilization rate after ICSI. However, all of the oocytes without visible spindles are not always abnormal. It is true that the normal fertilization and blastocyst rates of oocytes without visible spindles are lower, but we have witnessed the birth of two healthy babies after transfer of frozen-thawed blastocysts which did not have visible spindles.

In addition to the detection of visible spindles, the relationship between quantitative or qualitative criteria, such

Table 2. Effect of age on spindle parameters of human oocytes

Parameter	Under 35 years	36–40 years	Over 41 years
No. of oocytes	83	161	229
Spindle length (μm)	13.7 ± 1.4[a]	13.9 ± 2.0[a]	14.9 ± 2.3[b]
Spindle width (μm)	8.8 ± 1.1	9.0 ± 8.8	9.1 ± 1.2
Spindle area (μm^2)	97.6 ± 16.3[a]	102.8 ± 22. 3[a]	110.8 ± 25.6[b]
Spindle retardance (nm)	2.07 ± 0.47[a]	1.92 ± 0.46[a]	1.78 ± 0.45[b]

Oocytes were collected in letrozole-stimulated cycles. All oocytes were matured after they were retrieved. Differences between treatment groups were compared using one-way ANOVA.

as area, configuration and retardance on the spindle image, and the quality of oocytes (for example developmental competence) has been investigated. Kilani *et al.* [36] reported that a spindle was considered normal if it was complete, barrel-shaped and showed strong birefringence, and any other shape was considered abnormal. Spindle normality in the oocytes of their pregnant patients was significantly higher than that in pregnancy failure (100% vs. 33%, $P < 0.05$). Also, the spindle density, i.e. retardance, was significantly higher in the oocytes resulting in pregnancy; and fertilization rates, good-quality blastocyst rates, implantation rates and live birth rates of the oocytes with normal spindles were also higher than their respective rates in the oocytes with abnormal spindles. Spindle assessment with PolScope provides an early indication of the pregnancy potential of a particular oocyte. Therefore, spindle assessment should improve the selection of the best embryo for single embryo transfer [37].

A few studies have reported the relationship between spindle morphology by PolScope and embryo development. Spindle area, lengths and retardance were significantly greater in an early cleaving group (<27 h post-ICSI), and blastocyst rates and pregnancy rates in this group were significantly higher than those in a late cleaving group [38].

We analyzed the relationship between biological aging and spindle configuration in oocytes, and found that the areas, lengths and retardance of spindles in oocytes becomes greater with age (Table 2). Wang *et al.* [25, 35] observed under confocal microscopy that the abnormality of spindles in oocytes occurred with higher frequency in subjects aged from 40 to 45 years [39].

Observation of spindles in oocytes by PolScope is expected to become a method for the selection of high quality and viable embryos. However, spindles in oocytes are affected by temperature [40], pH [24], and the progress of meiotic maturation, and there are few reports of a consistent relationship between the spindle morphology and embryonic development or pregnancies after single embryo transfer. For clinical use, further studies will be needed to accurately evaluate the oocyte quality by spindle imaging and predict the viable embryos which give rise to successful pregnancies and births.

Improvement of Oocyte Quality Using Spindle Imaging

As noted above, it is thought that spindle images of the oocytes are one criterion for the determination and selection of good quality oocytes. In human ART, preovulatory oocytes are recovered from comparatively large follicles, but the maturity of oocytes is variable. The range of oocyte maturities may depend on meiotic maturation progress or oocyte aging in antral follicles.

In mice, the developmental competence of ovulated oocytes 15 h after hCG injection is very high, and the oocytes are defined as young. Observations of a meiotic spindle by PolScope and by fluorescence microscopy are respectively shown in Fig. 3A and Fig. 3D. The area of the spindles (Fig. 4A) in the young oocytes was 202.3 ± 4.6 μm^2 (mean ± SEM), and after the oocytes had been cultured for 8 h *in vitro*, i.e. 23 h after hCG injection, the area of the spindles of the aged oocytes had increased to 229.3 ± 4.4 μm^2(Fig. 4A), an area significantly greater than that of the young oocytes (Fig. 3B and E). When 5 μM MG132 was added to the medium for 8 h (Fig. 3C and F), the area of the spindles in the aged oocytes, 201.0 ± 4.5 μm^2, was smaller than that of the oocytes that were not treated with MG132, and similar to that of the young oocytes at 15 h after hCG injection (Fig. 4A). MG132 is an ubiquitin-mediated inhibitor of proteasome proteolysis and prevents MPF inactivation by blocking cyclin B degradation.

The blastocyst rate (Fig. 4B) of the aged oocytes (23 h post-hCG: cultured for 8 h) after ICSI significantly decreased compared to that of the young oocytes (60.3% vs. 93.3%). However, treatment with MG132 for 8 h im-

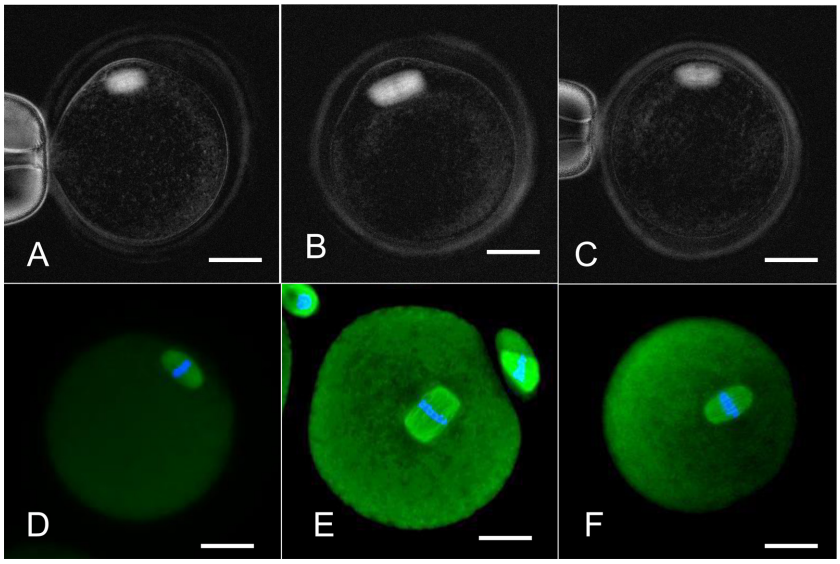

Fig. 3. Observation of a spindle in a mouse oocyte by PolScope (A-C) and fluorescent microscopy (D-F). Spindles in ovulated oocytes 15 h after hCG (young oocytes: A and D); spindles in oocytes 23 h after hCG injection (*in vitro* culture for 8 h: aged oocytes, B and E); spindles in MG132-treated oocytes (C and F). With oocyte aging, the area of the spindle becomes gradually greater, but the spindle area in the MG132-treated oocyte decreased. Scale is 20 μm.

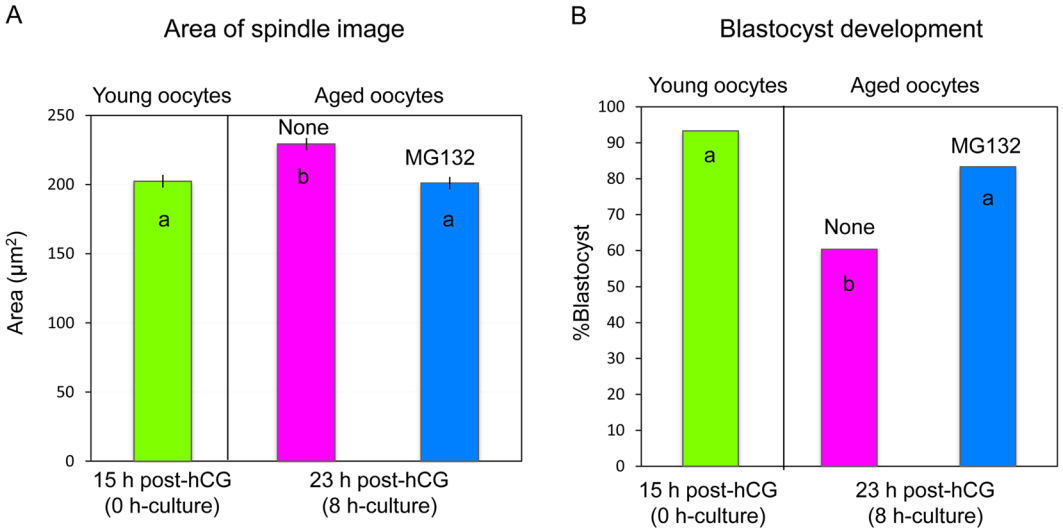

Fig. 4. Spindles in mouse oocytes with oocyte aging (A) and blastocyst development after ICSI (B). MG132 treatment of the aged oocytes resulted in a smaller spindle area (A) and increased the blastocyst rate (B). [a,b] $P < 0.05$.

proved the blastocyst rate of aged oocytes to a high rate (83.3%).

The area of spindles increases gradually with oocyte aging, and the blastocyst rate of the oocytes decreases with aging after ICSI. We showed that the addition of MG132 affected the spindle size in mouse oocytes after *in vitro* culture and improved the developmental competence of the oocytes [41]. Ono *et al.* [42] reported that MG132 maintained MPF activity, and that caffeine maintained normal meiotic spindle morphology; and they

obtained live offspring from BDF1 mouse oocytes transferred at 34 h after hCG injection.

There have been several reports of the reversibility of oocyte aging [43]. At present, it is difficult to change aged oocytes into young oocytes. However, the aging speed of oocytes may be delayed by reinforcement of MPF with, for example, MG132. Also, caffeine can delay porcine oocyte aging by regulating MPF [44]. The addition of melatonin maintains the developmental competence of oocytes with increasing age by preventing the formation of reactive oxygen species (ROS) [45].

The relationship between the meiotic spindle and the development competence of the oocytes is not yet clear, but it is thought that the spindle configuration and chromosome arrangement reflect the physiological stage or quality of the oocytes. Thus, the spindle image provides a useful index for the exploration of the optimal culture condition. Further investigations will be needed to determine the culture system for the improvement of oocyte quality and the production of high quality embryos.

Conclusion

The confirmation of the spindle location in the oocytes by polarized light microscopy has markedly reduced damage to spindles and chromosomes in ICSI. Also, when the presence of spindles in oocytes is not confirmed, the developmental competence of the oocytes is lower than that in the presence of a spindle. The detailed analysis of meiotic spindles (for example, area, length, and retardance), may provide more information for the evaluation of the developmental competence of oocytes.

In eSET, the criteria for the selection of quality embryos are important, and the assessment of meiotic spindles in oocytes by PolScope (a polarized light microscope) may indicate the optimal timing for ICSI. However, it is not an absolute marker, and it requires the support of other evaluation methods, for example, time-lapse monitoring. Further investigation is needed to demonstrate the relationships between spindle morphology and the developmental competence of oocytes, and their ability to develop to the blastocyst.

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