


## ORIGINAL ARTICLE

## Meiotic spindle size is a strong indicator of human oocyte quality

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## Abstract

**Purpose:** To investigate the relationship between the meiotic spindle size in human metaphase II oocytes and embryo developmental potential after intracytoplasmic sperm injection (ICSI).

**Methods:** Analyzed were 1302 oocytes with a visible meiotic spindle from 281 patients aged under 40 years undergoing ICSI cycles. The meiotic spindle was imaged by using PolScope before ICSI. The oocytes were classified into three groups, according to spindle size: group A (<90  $\mu\text{m}^2$ ), group B (90–120  $\mu\text{m}^2$ ), and group C (>120  $\mu\text{m}^2$ ).

**Results:** Overall, 389 (29.9%) oocytes were classified into group A, 662 (50.8%) into group B, and 251 (19.3%) into group C. The fertilization rate of the group B oocytes was significantly higher than for the A and C oocytes. The blastocyst formation rate in group B was significantly higher than in group A. In addition, the pregnancy rate in group B was significantly higher than in the other two groups.

**Conclusion:** The oocytes with a spindle size of 90–120  $\mu\text{m}^2$  showed higher fertilization, blastocyst formation, and clinical pregnancy rates than those with larger or smaller spindles. The measurement of the meiotic spindle size thus has a positive predictive value for identifying human embryo developmental potential clinically.

## KEYWORDS

assisted reproductive technology, blastocyst, fertilization, intracytoplasmic sperm injection, meiotic spindle

## 1 | INTRODUCTION

The embryo quality and developmental potential are among the most important issues in modern human embryology. Embryos for embryo transfer usually are selected by using one of several embryo quality assessment methods after 1–6 days of cultivation.<sup>1–3</sup> These embryo quality assessment methods serve to identify patients with a good prognosis for pregnancy, regardless of whether their embryos are transferred on day 3 or day 5. However, few reports of the methods for assessing strong indicators of oocyte quality have been

published. Evaluating the quality of oocytes is also one of the most important issues in assisted reproductive technology (ART). The oocyte quality can be a major factor in determining the fertilization of oocytes and their development to high-quality embryos, as well as for treating infertility. It has been reported that visualization of the meiotic spindle might be useful for the evaluation of oocyte quality.<sup>4</sup> The spindle apparatus is an essential cellular organelle, crucial for the high fidelity of chromosomal segregation during the meiotic divisions in oocyte maturation.<sup>5</sup> The meiotic spindle has been investigated extensively as a possible predictive feature for oocyte

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selection. Classically, the meiotic spindle has been visualized by fluorescence microscopy, which offers reliable and detailed information on the microtubular structures and associated chromosomes. However, its clinical use is limited by its invasive nature and its inability to be used for studying live oocytes. Approximately 15 years ago, imaging of the meiotic spindle in live oocytes was made possible by the development of an orientation-independent polarized light microscope, PolScope, which allows the uninvase study of spindle architecture in live human oocytes, without affecting their viability.<sup>4,6</sup> No detrimental effect on oocyte maturation or subsequent embryonic development was identified when PolScope was used to image mouse and human oocytes.<sup>7,8</sup> Using this technique, some meiotic spindle parameters were examined in relation to oocyte developmental potential after intracytoplasmic sperm injection (ICSI)<sup>9-12</sup> and the use of spindle imaging as a marker for the optimal timing of ICSI was investigated.<sup>13</sup> These studies indicated that visualization of the meiotic spindle might be useful in the evaluation of oocyte quality. However, there has been no report about the meiotic spindle size in human oocytes. The authors' previous study indicated that the meiotic spindle characteristics are significantly associated with early- or late-cleaving embryos.<sup>14</sup> Thus, quantitative measurement of meiotic spindle parameters might be valuable in identifying human oocyte and embryo developmental potential in ART laboratories. Against this background, in this study, it was aimed to investigate the association between the meiotic spindle size in human oocytes and embryonic developmental competence after ICSI.

## 2 | MATERIALS AND METHODS

### 2.1 | Patients

Analyzed were 1302 oocytes with a visible meiotic spindle from 281 patients under the age of 40 years who were undergoing ICSI cycles. Their mean age was  $36.5 \pm 2.6$  years ( $\pm$  standard deviation; range: 27-39). The etiologies of infertility in the cycles included male factor, tubal, uterine, diminished ovarian reserve, unexplained, or combined. The distribution of these etiologies was similar among the groups that were established, as described below. The study protocol and the need to perform ICSI and PolScope imaging analysis were explained to the couples and they were recruited into the

study after their approval. This study was approved by the Ethics Committee of IVF Nagata Clinic, Fukuoka, Japan.

### 2.2 | Stimulation and oocyte retrieval

Ovarian stimulation was achieved by a short protocol using a gonadotropin-releasing hormone agonist. Follicular growth was stimulated by injecting human menopausal gonadotropin (Gonapure; ASKA Pharmaceutical, Tokyo, Japan). In a few cycles, the minimal stimulation protocol was used. The minimal stimulation was started with an extended regimen of clomiphene citrate in conjunction with low-dose gonadotropin injections. The ovarian response was monitored by a daily measurement of the follicular diameter. When at least one or two follicles had reached  $\geq 18$  mm in diameter, an injection of 10 000 IU of human chorionic gonadotropin (hCG) (Fuji Pharma Company, Tokyo, Japan) was given i.m. to mimic the normal luteinizing hormone surge. The oocytes were recovered 35-36 hours after the administration of hCG, following transvaginal ultrasound-guided puncture of the follicles. After brief exposure to 80 IU/mL hyaluronidase solution (Origio, Copenhagen, Denmark), cumulus cells were removed by repeated gentle pipetting. The denuded oocytes then were assessed with respect to their meiotic maturation status. In preparation for ICSI, the oocytes with an extruded first polar body (PB1) (presumably at the metaphase II stage) were selected for meiotic spindle detection and PB1 morphology assessment. The oocytes without a polar body or presenting a germinal vesicle were excluded from this study. Then, ICSI was performed 40-41 hours after hCG administration.

### 2.3 | Visualization of the spindle using PolScope and intracytoplasmic sperm injection

In this study, visualization of the spindle using PolScope and ICSI were performed as in the authors' previous study.<sup>14</sup> For spindle imaging, each oocyte was placed in a 10  $\mu$ L drop of HEPES-buffered medium (Global with HEPES; Life Global, Guelph, ON, Canada), covered with sterile mineral oil (Fuso Pharmaceutical, Osaka, Japan) on a glass-bottomed culture dish (FluoroDish; World Precision Instruments, Inc., Sarasota, FL, USA), which was maintained at 37°C on a heated stage. The oocytes were imaged using an Olympus IX72 inverted microscope that was equipped with the polarized light LC PolScope

**TABLE 1** Spindle characteristics of the metaphase II (MII) oocytes that were observed using PolScope

Parameter	Spindle size ( $\mu\text{m}^2$ )		
	A: <90	B: 90-120	C: >120
No. of MII oocytes	389	662	251
Oocyte diameter ( $\mu\text{m}$ ) <sup>a</sup>	$109.9 \pm 4.7\text{a}$	$111.3 \pm 5.4\text{b}$	$112.0 \pm 5.7\text{b}$
Spindle-to-polar body angle ( $^\circ$ ) <sup>a</sup>	$23.6 \pm 26.6\text{a}$	$25.1 \pm 23.1\text{a}$	$32.1 \pm 30.2\text{b}$
Spindle length ( $\mu\text{m}$ ) <sup>a</sup>	$12.2 \pm 1.5\text{a}$	$14.2 \pm 1.3\text{b}$	$16.7 \pm 1.8\text{c}$
Spindle light retardancy (nm) <sup>a</sup>	$2.3 \pm 0.7\text{a}$	$2.4 \pm 0.7\text{b}$	$2.0 \pm 0.6\text{c}$

<sup>a</sup>Values are the mean  $\pm$  standard deviation.

Within the same row, values with different superscripts are significantly different ( $P < .05$ ).

(CRi, Woburn, MA, USA), combined with a computerized image analysis system (Oosight imaging system; CRi). The microscope was set up and the background was calibrated in accordance with the manufacturer's protocol. The spindle images were acquired at 400× magnification and the location, area (size), and length of the meiotic spindle (in  $\mu\text{m}$ ) and the mean light retardancy (in nm) by the birefringent spindles were recorded in the image processing system for later analysis. To optimize visualization of the spindle and the polar body, the oocytes were rotated by using a holding and injection pipette. Additionally, a maximum area of meiotic spindle with a spindle shape was imaged. After imaging, ICSI was performed with the spindle at the 6 or 12 o'clock position. After ICSI, the oocytes were washed and cultured separately in droplets of Global medium (Life Global) that was supplemented with 20% substitute serum (NPS; Nakamedical, Tokyo, Japan) in order to examine fertilization.

## 2.4 | Classification of the meiotic spindle size

The meiotic spindle characteristics were imaged using PolScope before ICSI. After imaging, the spindle size was measured with the Oosight imaging system. Then, the oocytes were classified into three groups, according to the size of their meiotic spindle: A ( $<90 \mu\text{m}^2$ ), B ( $90\text{--}120 \mu\text{m}^2$ ), and C ( $>120 \mu\text{m}^2$ ).

## 2.5 | In vitro culture of the human embryos

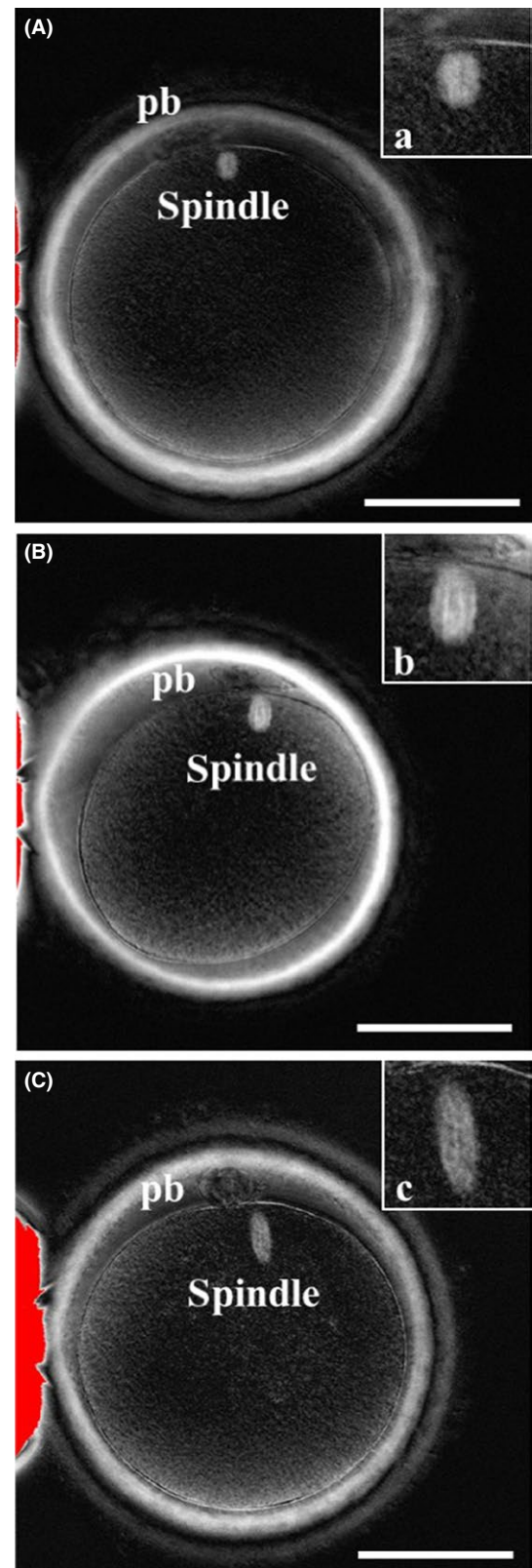
After ICSI, all the oocytes were cultured separately in Embryo GPS dishes (Sun IVF, Guilford, CT, USA) with Global medium (Life Global) at 37°C in an atmosphere of 5%  $\text{O}_2$ , 5%  $\text{CO}_2$ , and 90%  $\text{N}_2$ . The oocytes were monitored by using a time-lapse incubator (CCM-IVF and iBIS; Astec, Fukuoka, Japan) to determine pronuclear formation and cleavage divisions. During culture in the time-lapse incubator, digital images of the cultured embryos were taken at 20 min intervals. Image stacks were analyzed by using CCM-IVF software (ASBRO; Astec) and CCM-iBIS software (Photo Tunes; Astec). The oocytes with two pronuclei and a second polar body were considered to be normally fertilized. The zygotes were cultured in 30  $\mu\text{L}$  droplets of Global medium (Life Global) for 2 days post-ICSI. Some zygotes were cultured in Global medium separately for 5–6 days and were evaluated for their rate of development to the blastocyst stage.

## 2.6 | Definition of good-quality embryos

The embryos that had reached the two-cell stage by 27 hours post ICSI and had good morphological scores ( $\geq 4$  cells with  $<50\%$  fragmentation) on day 2 were defined as good-quality embryos on day 2. The blastocysts that had obtained a score of 3BB or more by using Gardner's scale<sup>3</sup> were defined as good-quality blastocysts.

## 2.7 | Single-embryo transfers

In this study, the clinical pregnancy rates of the three groups were compared by using fresh and vitrified-warmed cycles. In the fresh



**FIGURE 1** Meiotic spindle of human oocytes, as imaged by PolScope. The oocytes were classified into three groups, according to their spindle size: (A)  $<90 \mu\text{m}^2$ , (B)  $90\text{--}120 \mu\text{m}^2$ , and (C)  $>120 \mu\text{m}^2$ . The spindle image is shown at higher magnification in the insets (a–c). pb, the first polar body. Scale bar = 50  $\mu\text{m}$

**TABLE 2** Fertilization, cleavage, and blastocyst rates of metaphase II (MII) oocytes with different spindle sizes after intracytoplasmic sperm injection

Parameter	Spindle size ( $\mu\text{m}^2$ )		
	A: <90	B: 90-120	C: >120
MI I oocytes (N)	389	662	251
Normal fertilized oocytes (%)	294 (75.6)a	555 (83.8)b	187 (74.5)a
Abnormal fertilized oocytes (%)	23 (5.9)a,b	31 (4.7)a	21 (8.4)b
Degenerated oocytes (%)	15 (3.9)	17 (2.6)	6 (2.4)
Cleaved embryos (%) <sup>a</sup>	283 (96.3)	542 (97.7)	178 (95.2)
Good-quality embryos on day2 (%) <sup>a</sup>	85 (28.9) a	289 (52.1)b	111 (59.4)b
Blastocyst cultured embryos (N)	96	194	76
Blastocysts (%)	39 (40.6)a	103 (53.1)b	40 (52.6)a,b
Good-quality blastocysts (%)	20 (20.8)	57 (29.4)	22 (28.9)

<sup>a</sup>Per normal fertilized oocyte.

Within the same row, values with different superscripts are significantly different ( $P < .05$ ).

cycles, the embryo transfer was performed by using cleavage-stage embryos. In the vitrified-warmed cycles, the embryo transfer was performed by using blastocyst-stage embryos. The vitrified-warmed embryo transfer was performed by using the hormone replacement therapy cycle. In this study, the clinical pregnancy rates in the three groups were summarized when only one embryo was transferred. "Clinical pregnancy" was defined as the presence of an intrauterine gestational sac that was seen at an ultrasonographic examination 3-4 weeks after the embryo transfer.

## 2.8 | Statistical analysis

The statistical analysis was performed by using GraphPad Prism 5.0 software (GraphPad, Inc., San Diego, CA, USA). For comparisons among groups, the Student's *t* test was used for the continuous variables and the chi-square test was used for the binary variables. The differences were considered to be significant when  $P < .05$ .

## 3 | RESULTS

### 3.1 | Meiotic spindle characteristics of the oocytes with spindle imaging using PolScope

Among the 1302 oocytes with a visible spindle, 389 (29.9%) were classified as having a spindle size of  $<90 \mu\text{m}^2$  (group A), 662 (50.8%) as  $90-120 \mu\text{m}^2$  (B), and 251 (19.3%) as  $>120 \mu\text{m}^2$  (C). Table 1 presents the spindle characteristics of the oocytes in these three groups and Figure 1 shows representative images of the spindles. The spindle image in group B (Figure 1B,b) was more distinct than those in groups A and C (Figure 1A,a; C,c). The oocyte diameter in the group A oocytes was significantly smaller ( $P < .01$ ) than in the B and C oocytes ( $109.9 \pm 4.7 \mu\text{m}$  vs  $111.3 \pm 5.4 \mu\text{m}$  and  $112.0 \pm 5.7 \mu\text{m}$ , respectively). The spindle-to-polar body angle in the group C oocytes was significantly greater ( $P < .01$ ) than in the A and B oocytes ( $32.1 \pm 30.2^\circ$  vs  $23.6 \pm 26.6^\circ$  and  $25.1 \pm 23.1^\circ$ , respectively). The spindle length was also proportional to size. Moreover, the spindle light retardancy in

the group B oocytes was significantly greater ( $P < .05$ ) than in the A and C oocytes ( $2.4 \pm 0.7 \text{ nm}$  vs  $2.3 \pm 0.7 \text{ nm}$  and  $2.0 \pm 0.6 \text{ nm}$ , respectively).

### 3.2 | Fertilization and blastocyst formation rates of the oocytes with different spindle sizes

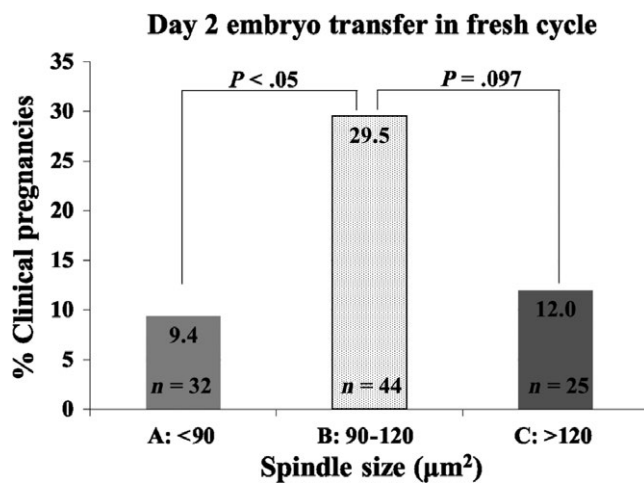
Table 2 presents the fertilization, cleavage, and blastocyst rates of the three groups after ICSI. There was no significant difference in the degeneration of the oocytes and cleavage embryos among the three groups. The rate of normal fertilization in the group B oocytes was significantly higher ( $P < .01$ ) than in the A and C oocytes (83.8% vs 75.6% and 74.5%, respectively). The rate of abnormal fertilization in the group C oocytes was significantly higher ( $P < .05$ ) than in the B oocytes (8.4% vs 4.7%). The proportion of embryos that developed into good-quality embryos on day 2 in group B was significantly higher ( $P < .01$ ) than that in group A (52.1% vs 28.9%). In order to evaluate the competence to develop to the blastocyst stage, 96 zygotes in group A, 194 in group B, and 76 in group C were cultured in Global medium. The proportion of embryos that developed to the blastocyst stage in group B was significantly higher ( $P < .05$ ) than that in group A (53.1% vs 40.6%).

### 3.3 | Clinical pregnancy rates after a single-embryo transfer

Table 3 presents the patients' characteristics among the three groups who had a fresh early embryo transfer and Figure 2 shows the associated clinical pregnancy rates. There was no significant difference in the patient characteristics among the three groups. However, the proportion of clinical pregnancies in group B was higher than in groups A and C (29.5% vs 9.4% and 12.0%, respectively). Table 4 presents the patients' characteristics among the three groups who had a vitrified-warmed blastocyst transfer and Figure 3 shows the associated clinical pregnancy rates. There was no significant difference in patient characteristics among the three

**TABLE 3** Patients' characteristics among the three groups who had a fresh early embryo transfer

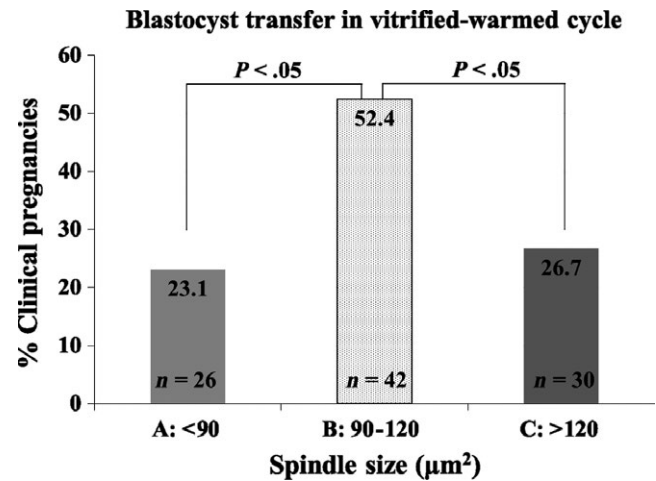
Parameter	Spindle size ( $\mu\text{m}^2$ )		
	A: <90	B: 90-120	C: >120
No. of embryo transfer cycles	32	44	25
Female age (years) <sup>a</sup>	36.0 $\pm$ 2.3	36.6 $\pm$ 2.8	37.1 $\pm$ 2.0
Endometrial thickness (mm) <sup>a</sup>	9.9 $\pm$ 1.7	10.0 $\pm$ 2.1	9.5 $\pm$ 1.8
No. of transferred embryos <sup>a</sup>	1.0 $\pm$ .0	1.0 $\pm$ .0	1.0 $\pm$ .0
Good-quality embryo transfer cycles: N (%)	14 (43.8)	28 (63.6)	17 (68.0)

<sup>a</sup>Values are the mean  $\pm$  standard deviation.**FIGURE 2** Pregnancy rates from the early human embryos that were derived from the three groups of metaphase II oocytes. The actual percentages are shown within the bars**TABLE 4** Patients' characteristics among the three groups who had a vitrified-warmed blastocyst transfer

Parameter	Spindle size ( $\mu\text{m}^2$ )		
	A: <90	B: 90-120	C: >120
No. of embryo transfer cycles	26	42	30
Female age (years) <sup>a</sup>	35.5 $\pm$ 1.8	36.3 $\pm$ 3.1	35.7 $\pm$ 3.8
Endometrial thickness (mm) <sup>a</sup>	10.3 $\pm$ 2.3	10.2 $\pm$ 1.9	10.7 $\pm$ 1.9
No. of transferred embryos <sup>a</sup>	1.0 $\pm$ 0.0	1.0 $\pm$ 0.0	1.0 $\pm$ 0.0
Good-quality blastocyst transfer cycles: N (%)	12 (46.2)	22 (52.3)	14 (46.7)

<sup>a</sup>Values are the mean  $\pm$  standard deviation.

groups. However, the proportion of clinical pregnancies in group B was higher than in groups A and C (52.4% vs 30.8% and 26.7%, respectively).

**FIGURE 3** Pregnancy rates from the human blastocysts that were derived from the three groups of metaphase II oocytes. The actual percentages are shown within the bars

## 4 | DISCUSSION

The present study confirmed that spindle birefringence in living human oocytes can be imaged using PolScope and provide quantitative morphological information. In this study, the size of the meiotic spindle was quantified by using PolScope and was classified into three groups, according to the cross-sectional area of the meiotic spindle. A meiotic spindle size of 90-120  $\mu\text{m}^2$  predicted not only a higher fertilization rate, but also a higher blastocyst formation rate. This confirmed that there is a close relationship between the size of the meiotic spindle in human metaphase II (MII) oocytes and embryo developmental potential after ICSI. Several studies also reported that some spindle characteristics of MII oocytes, observed using PolScope, are associated with a higher fertilization rate and embryo development.<sup>15-17</sup> One meta-analysis also led to the conclusion that the presence of a birefringent meiotic spindle in human oocytes predicts a better fertilization rate and embryo development.<sup>18</sup> No difference was found for pregnancy or implantation rates. In contrast, this study showed that the oocytes with a meiotic spindle size of 90-120  $\mu\text{m}^2$  have high implantation capacity and that the meiotic spindle size of the MII oocytes was revealed to be a novel marker for evaluating oocyte quality. In contrast, it previously was reported that the mean meiotic spindle area did not differ significantly between transfer oocytes from conception and non-conception cycles.<sup>19</sup> This study indicated that oocytes with spindles outside the optimal size range (<90  $\mu\text{m}^2$  or >120  $\mu\text{m}^2$ ) produced embryos that were associated with a lower clinical pregnancy rate. From the above, it was thought that the spindle size in the non-conception cycle was polarized into small and large spindles. Therefore, it is thought that the mean area of the meiotic spindle in non-conception cycles becomes similar to that in conception cycles.

Another experiment showed that light retardancy measurements have limited value for predicting the degree of spindle fiber order



and chromosomal position<sup>20</sup>; moreover, the results of a morphometric evaluation of the spindle through PolScope were not consistent with the findings of a confocal analysis. Therefore, it was concluded in the literature that PolScope only provides a qualitative observation, rather than a quantitative one. However, it might not have been possible to visualize the meiotic spindles well using PolScope in that study.<sup>20</sup> The meiotic spindles of most mammals, including humans, are very sensitive to environmental changes, especially to fluctuations in temperature,<sup>21,22</sup> which is thus an important parameter that affects the integrity of the spindle during the handling and culturing of oocytes in vitro. A change in the ambient temperature can induce temporary depolymerization of the microtubules.<sup>23</sup> In the present study, a heating system was used during oocyte examination and ICSI in order to maintain the temperature of the medium at 37°C. Therefore, temperature fluctuations were unlikely to have had a significant impact on the size of the meiotic spindle. However, the spindle light retardancy in the oocytes in this study was greater than in a previous study<sup>20</sup> (range: 2.0–2.4 nm vs 0.83–1.46 nm, respectively). This suggested that, in the current study, the meiotic spindle of the MII oocytes was efficiently visualized using PolScope. In addition, a prospective observational study reported that the assessment of normality of the meiotic spindle has a strong value in predicting the likelihood of pregnancy for a particular oocyte.<sup>24,25</sup>

It has been reported that there is a positive correlation between the spindle light retardancy and embryonic developmental potential.<sup>12</sup> The pregnancy rate also was found to be strongly correlated with the light retardancy of the meiotic spindle in human oocytes.<sup>17</sup> Here, it also was found that the light retardancy of the meiotic spindle in the oocytes with a meiotic spindle size of 90–120  $\mu\text{m}^2$  was significantly greater than in the oocytes of the other sizes. This confirmed the existence of an association between the meiotic spindle size and the light retardancy of human oocytes. Thus, the meiotic spindle size has a strong influence on the embryonic developmental potential after ICSI.

This study showed that the blastocyst formation rate was similar between oocytes with meiotic spindle sizes of 90–120  $\mu\text{m}^2$  and >120  $\mu\text{m}^2$ . However, the proportion of clinical pregnancies in relation to the oocytes with a meiotic spindle size of 90–120  $\mu\text{m}^2$  was higher than for the oocytes with a meiotic spindle size of >120  $\mu\text{m}^2$ . It is known that the implantation rate of aneuploidy embryos is low. Therefore, the rate at which euploid embryos develop from oocytes with a spindle size of 90–120  $\mu\text{m}^2$  might be higher than those for oocytes of other sizes. In actuality, it has been reported that oocytes with normal spindle morphology are significantly more likely to produce euploid embryos than oocytes with meiotic spindles that are translucent or not visible.<sup>26</sup> In the future, the authors would like to experimentally confirm the association between the meiotic spindle size and embryo ploidy.

There are two possibilities as to why the outcome of oocytes with large or small meiotic spindles became poor. First, the polymerization and depolymerization dynamics of the microtubules have been reported to contribute to the control of the size and shape of the spindles.<sup>27,28</sup> Thus, the size of the meiotic spindle is

controlled by cytoplasmic factors that regulate the microtubular dynamics and it could be that factors associated with cytoplasmic maturation, such as mitochondria, are not normal in the oocytes with large or small spindles. In mouse experiments, the authors have shown that the adenosine triphosphate (ATP) level of oocytes with large or small spindles was significantly lower than that of the oocytes with a middle-range spindle size (unpublished data). The ATP is generated by the mitochondria. The oocytes with large or small spindles were considered to be of poor quality related to their mitochondrial function or numerical aberration. Second, in this study, meiotic spindle observation and ICSI were performed at almost the same time after hCG administration in all cases, but the meiotic spindle size could change with the duration of the in vitro culture. Actually, in the mouse experiment, it was clarified that the meiotic spindle size increased as the time elapsed after hCG administration (unpublished data). Thus, oocytes with large or small meiotic spindles could be improved by optimizing the timing of the oocyte pick-up or performance of ICSI. The authors will consider this point in the future and will investigate whether the meiotic spindle size will change, depending on the oocyte maturation period.

In conclusion, MII oocytes with a spindle size of 90–120  $\mu\text{m}^2$  produced embryos with a higher rate of blastocyst formation and higher subsequent pregnancy rate than did the oocytes with spindles outside of that size range. These results suggest that the quantitative measurement of the meiotic spindle size would have a positive predictive value for identifying human embryo developmental potential in infertility clinics.

## DISCLOSURES

**Conflict of interest:** The authors declare no conflict of interest.  
**Human Rights Statement and Informed Consent:** All the procedures were followed in accordance with the ethical standards of the institutional ethical committee and with the Helsinki Declaration of 1964 and its later amendments. All the study's participants provided informed consent and the study design was approved by the appropriate ethics committee of IVF Nagata Clinic, Fukuoka, Japan.  
**Animal studies:** This article does not contain any study with animal participants that have been performed by any of the authors.

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