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Location of second oocyte spindle and ART outcome

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Location of second oocyte spindle and ART outcome

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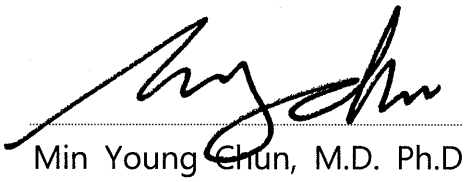
Committee Member 

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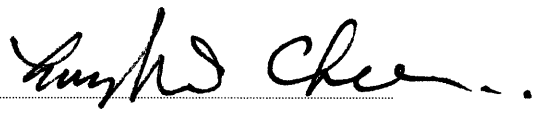
Committee Member 

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ABSTRACT

Location of second oocyte spindle and ART outcome

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Dysregulated oocyte maturation can cause chromosomally impaired development and it has been emerged as an infertility biomarker. Disrupting of chromosomal segregation can be occurred with applying the artificial reproductive technology. However, the relationship between the location of the oocyte meiotic spindle and embryo development including chromosomal abnormality has yet to be well-studied in the in vitro fertilization (IVF) cycles. Therefore, this study investigated the association between the angle of meiotic spindle deviation and embryo development and euploidy in PGT-A cycle patients. Morphokinetic analysis was performed with a time-lapse monitoring system. This study was conducted on 2441 oocytes from 303 patients who underwent Polscope and PGT-A from March 2022 to January 2023. Spindle in oocytes was observed using Polscope before intracytoplasmic sperm injection (ICSI), and oocytes were divided into 4 groups according to the angle of meiotic spindle deviation from the polar body position: A1 (beneath; 0-5^{o}), A2 (adjacent; 5-15^{o}), A3 (away; >15^{o}), and A4 (non-visible).

The embryos were single-cultured according to oocyte meiotic spindle position and monitored using the time-lapse system to assess embryo developmental competence. Fertilization rate was significantly low in the non-visible spindle A4 group compared with other groups (A1 vs. A4; p = 0.0005, A2 vs. A4; p = 0.0002, A3 vs. A4; p = 0.0003). The good quality embryo (GQE) rate by each group showed numerical values of A1 (86.8%), A2 (86.2%), A3 (83.6%), and A4 (77.8%). Among groups with a spindle, only A1 and A2 showed significant differences in the GQE rate from the non-visible group (A1 vs. A4; p = 0.0017, A2 vs. A4; p = 0.0066). Also, the groups having a spindle showed statistically higher blastocyst rates of biopsy than those in the group without a spindle on Day 5 or 6 (A1 vs A4; p = 0.0005, A2 vs.A4; p = 0.0002, A3 vs A4; p = 0.0002). Although there were no statistically significant differences in aneuploidy rate by the spindle position, it showed sequentially increased numerical values from A1 (n=197, 59.0%), A2 (n=129, 59.2%), A3 (n=129, 63.5%), to A4 (n=37, 64.9%). The effect of relative spindle position on morphokinetic parameters of euploid, aneuploid and mosaic blastocysts was investigated. There was a significant difference between spindle angles in A1 and A2 at tPNf (24.6±2.9 vs 23.1±2.5) and t2 (27.1±3.0 vs 25.2±2.5) in the euploid embryo group. The results of mosaic embryo groups showed significant differences between spindle angles in A1 and A3 when t7 (58.5±9.1 vs 52.6±7.5) and t8 (62.7±10.7 vs 56.0±7.4). The relative position of the spindle within the oocyte impact fertilization, embryo development rates and the time-point of early embryo stage as a morphokinetic parameter in patients in PGT-A cycles. It suggested that the position of the oocyte meiotic spindle with morphokinetic parameters may provide essential information to identify euploid embryos with the highest developmental potential.

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INTRODUCTION

In recent fertility research, the main challenge is how to reduce the miscarriage rate and increase the pregnancy rate of patients and how to culture good embryos. Various methods are still being studied to increase the pregnancy rate, one of which is preimplantation genetic testing for aneuploidy (PGT-A).

PGT-A is a test to screen for normal embryos before fertilized embryos are implanted into the uterus. Patients undergoing PGT-A include followings : habitual miscarriage, repeated implantation failure (RIF), advanced maternal age (AMA), patients with chromosomal abnormalities in the past, patients with very poor sperm quality. In these patients, not only Down syndrome, Patau syndrome, and Edwards syndrome, which have three chromosomes, can be identified through PGT-A, but also numerical abnormalities of other chromosomes. This has the effect of increasing the pregnancy rate and lowering the miscarriage rate in embryo transfer. This is because if the number of chromosomes is one, it is difficult to become pregnant, and if there are three chromosomes, it becomes a miscarriage at the beginning of pregnancy. According to other studies, more than 60% of spontaneous abortions occurring in pregnancy are due to chromosomal abnormalities (Choi et al., 2014). PGT-A prevents miscarriage by screening and not transferring these embryos. Therefore, many IVF centers are screening embryos with PGT-A to reduce the rate of miscarriage caused by chromosomal abnormalities.

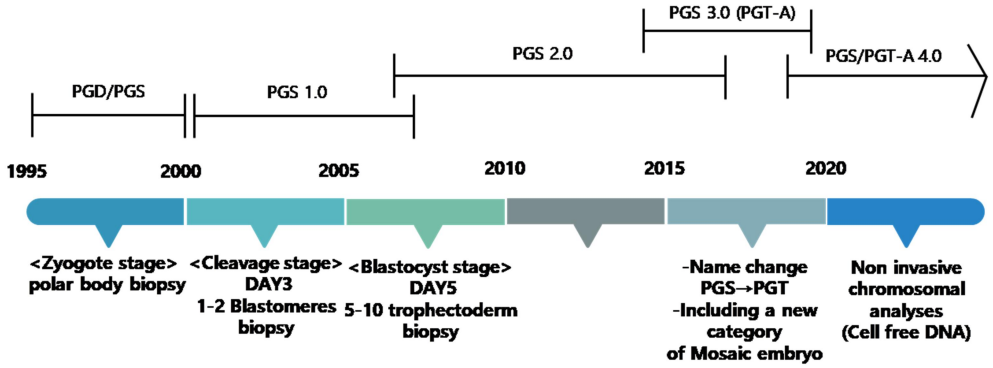

Figure 1. Advanced of Preimplantation Genetic Testing technique

The history of PGT is not that long. PGT began with preimplantation genetic diagnostics (PGD), which recognizes a single genetic disorder, and expanded to detect aneuploidy embryo and remove it before transfer (Gleicher et al., 2021). In the late 1990s, genetic testing was proposed for the first or second polar body at the zygote stage (Verlinsky et al., 2002), but it was hard to accept because it was technically difficult. Instead, day 3 after fertilization, one or two blastomere biopsy was performed, which was called preimplantation genetic screening (PGS) (Scriven et al., 2011). In 2008, it moved from cleavage stage to the blastocyst stage biopsy on the day 5 after fertilization (Jansen et al., 2008). In the blastocyst stage, 5-10 tropectoderm cells biopsied and analyzed. So it can provide a larger amount of DNA from the tropectoderm. In 2016, The preimplantation genetic diagnosis international society (PGDIS) published the first practical guidelines for PGS on its website. At the same time, the name of Procedure/TEST was changed from PGS to PGT. Most importantly, we added a new third category of mosaicism embryos, rather than just classifying embryos into euploid and aneuploidy binaries (Gleicher et al., 2017). Until now, single trophectoderm (TE) biopsy could not diagnose mosaicism in most diagnostic methods used

for PGS/PGT-A except for NGS. Therefore, the new PGDIS guidelines disqualified platforms other than NGS (Scott et al., 2016). Currently, the genetic testing industry is researching cell free DNA (cfDNA), that is, performing chromosome analysis through a non-invasive method. This can be obtained from embryo cultured media from 4-6 days, and chromosomal abnormalities can be identified by performing NGS in an amount of 10μl. It is expected to help clinical outcomes by avoiding damage caused by embryo biopsy (Huang et al., 2019). In order to use cfDNA as a clinical outcome, it must be representative of the total embryonic chromosome content. However, since there are many questions about this, further research in clinical and basic researches are needed (Rubio et al., 2020).

There are three types of PGT. The PGT includes testing for specific monogenic disorders (PGT-M), structural rearrangements of chromosomes (PGT-SR), or an abnormal number of chromosomes (i.e., aneuploidy (PGT-A)) (Pastore et al., 2019). The PGT-M avoids the ethically and emotionally difficult alternative of deciding whether to terminate a pregnancy that has been found to be affected by a genetic disorder. It also provides patients at risk of passing the inherited disease on to their offspring the ability to have children that do not have these effects. Chromosomal structural rearrangements (PGT-SR) include Robertsonian translocations, reciprocal or balanced translocations, and inversions. By examining these causes, infertility and recurrent pregnancy loss can be prevented (Insogna et al., 2021). The purpose of preimplantation genetic testing is to increase implantation and pregnancy rates by excluding aneuploidy embryos from transferring (Gleicher et al., 2021). PGT-A is a method for screening aneuploidy embryos by chromosome instability. This chromosome instability is caused by spindle

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disruption, which results in abnormal fertilization, fertilization failure, or rearrangement of chromosomes in oocyte cytoplasm (Moor et al., 1985 ; Aman et al., 1994).

The spindle that separating chromosome and others is a fibrous protein formed during the somatic cell division of a cell that connects the two poles or between the two poles and the chromosomes. After germinal vesicle (GV) breakdown, cytoplasmic microtubules break down and begin to form a large number of spindles around the chromosome. The function of these newly formed microtubules and other proteins causes the spindle to move toward of the oocyte cortex. This spindle causes half of the homologous chromosomes to be separated into polar body.

The oocyte meiotic spindle is a microtubule structure that is fundamental to meiosis and serves to align and separate chromosomes (Coticchio et al., 2004). Meiotic spindle is associated with the success of chromosome separation. It can support the efficiency of mechanisms such as sister chromatid cohesion stability, appropriate frequency and location of recombinant sites, telomere length, and spindle checkpoint control (Tsutsumi et al., 2014; Duncan et al., 2012). A failure of this mechanism or the spindle integrity itself can result in aneuploidy (Tilia et al., 2020). The chromosome segregation process is very sensitive to the timing of spindle formation or even small changes in biochemistry (Wasielak et al., 2022). Several studies have shown that naturally reproductive human oocytes from women older than 40 have a high incidence of meiotic spindle abnormalities, such as altered tubulin localization and chromosomal alterations in meiosis II

(Battaglia et al., 1996). In experiments with aging human oocytes in vitro, the integrity regulators of spindle stability regulators, such as microtubule-associated proteins, was also compromised (Hall et al., 2007). For this reason, aging of human oocytes increases the rate of spindle abnormality, which can lead to chromosomal abnormalities in the embryo. Thus, it is important to use a polarizing microscopy to determine the localization of the spindle and reduce damage to obtain successful clinical results.

In research related to IVF, the presence or absence of oocyte spindle gave a positive correlation in fertilization, embryonic development, and blastocyst formation. In addition, it has been suspected that embryos derived from oocytes with visible spindles have a higher probability of implantation and pregnancy rates, while non-visible spindle have significantly lower implantation and pregnancy rates. In one study, pregnancy and implantation rates were higher when the visible spindle group was transferred than the non-visible spindle group (PR: 44.4% vs 23.0%, IR: 18.2% vs 8.7%) (Madaschi et al., 2008).

We still have limited information on the correlation between oocyte meiotic spindle and PGT-A results. The clinical results according to the presence or absence of the spindle are also controversial. Therefore, in this study, PGT-A was conducted to analyze the genetic characteristics of embryos according to the presence and angle of the meiotic spindle of the human oocyte. The fertilization rate, embryo development rate, euploidy rate and the clinical outcomes according to the oocyte meiotic spindle were analyzed.

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MATERIALS AND METHODS

Patients

This was a retrospective cohort study from March 2022 to January 2023 evaluating the data from patients who were submitted to ART in the IVF center. This study was approved by CHA Fertility Center Gangnam, CHA University (Seoul, Korea). Patients undergoing ICSI for the purpose of performing blastocyst stage preimplantation genetic testing for aneuploidy (PGT-A) were included regardless of age or indication. Patients underwent freeze on day 5 or day 6, respectively. Biopsy was carried out when blastocysts reached the expanded stage on day5 or day6. Analysis of frozen embryo transfers, with PGT-A was done to evaluate the clinical outcomes (clinical pregnancy rate, miscarriage rate). PGT-M, PGT-SR cycles, oocyte recipients, surrogacy, and vitrified-warmed oocytes were excluded from this study (Fig. 1).

Ovum pick up and ICSI with spindle observation

Oocytes were retrieved and placed in a fertilization medium (Quinn's Advantage®, CooperSurgical, USA) and then the cumulus-corona complex was removed using enzymes and repeated pipetting, two hours after OPU. All cumulus-corona cells were stripped to reduce the possibility of maternal DNA contamination during biopsy. ICSI proceeded two hours after oocyte denudation. Mature oocytes were selected to reduce chromosomal damage by observing meiotic spindle using Polscope spindle view (LC-PolScope™, USA) before ICSI. A glass bottom dish (MatTEK, USA) is used to observe the

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oocyte meiotic spindle. All oocytes were performed ICSI after meiotic spindle observation. Theoocyte meiotic spindle angle was measured based on the 1st polarbody of the oocyte. The group was divided into A1 (beneath; 0-5º), A2 (adjacent; 5-15º), A3 (away; >15º), and A4 (non-visible) based on the polar body (PB) (Fig. 2).

Embryo Culture

Some embryos were cultured in conventional incubator (Heracell™, Thermo Fisher, MA, USA) and some embryos were incubated in Time-lapse incubator (EmbryoScope™, Vitrolife, Sweden), at 37°C with 6% CO_2 and 5% O_2. After ICSI, each oocyte individually cultured cleavage medium in 20μl droplet (Quinn's Advantage®, CooperSurgical, USA) and covered with mineral oil (OVOIL™ ,Vitrolife, Sweden). Fertilization was confirmed by the presence or absence of two pronuclei (2PN) after 16-18 hours of ICSI. On day 3, embryos were transferred to blastocyst medium and their development evaluated. The grade of good quality embryo is defined as 6-cell or more on day 3. Embryo development was assessed on day 5, and blastocyst biopsies were performed on adequately progressed embryos. If the blastocyst was not expanded on day 5, we biopsied on day 6.

Blastocysts were graded according to original Gardner grading system, which assessed the level of expansion (graded 1 to 6) as well as inner cell mass (ICM) and TE cells (graded A to C). Gardner grading system developed for assessing the degree of blastocyst expansion and cell number of TE and ICM.

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Blastocyst expansion rate was characterized into 6 types: 1, early blastocyst where the blastocyst cavity is less than half the embryo volume; 2, mid blastocyst, with a blastocyst cavity that is half or more than half of the embryo volume; 3, full blastocyst, with the blastocyst cavity completely filling the embryo; 4, expanded blastocyst, the blastocyst cavity volume is larger than early blastocyst within thinning of the zona pellucida; 5, hatching blastocyst, the trophectoderm cells begin to escape through the zona pellucida, and 6 hatched blastocyst where the blastocyst has completely escaped from the zona pellucida. The ICM was assessed as followed A, full of cells; B, loosely-grouped several cells; or C, very few cells. The TE was assessed as follow A, many cells that form a cohesive epithelium; B, a small number of cells forming a loose epithelium; C, very few cells (Gardner et al., 2000).

Trophectoderm Biopsy

Before biopsy, the blastocysts were graded based on the morphology of ICM, TE, as well as the expansion stage, according to the original Gardner grading system. Biopsy timing was performed when blastocysts reached the expansion stage for genetic analysis on day 5 or day 6. Embryos were transferred to individual biopsy plates with equilibrium microdrops of 10μl of 5% HSA/HEPES buffered medium (SAGE Quinn's-HEPES, CooperSurgical, Trumbull, CT, USA) covered with mineral oil (Oil for tissue culture, CooperSurgical, Denmark). After placing the ICM in the 6 o'clock position and fixing it with a holding pipette, and used a laser to drilling the zona pellucida of the blastocyst. After that, 5~10 TE cells were smoothly aspirated, and the junction of the trophectodem cell was cut using a laser, and then

separated from the blastocyst by the flicking method. Subsequently, the TE cell was washed 3 times in 1X PBS and tubing was performed in a PCR tube containing 2μl 1X PBS. The samples were stored at -20℃ and transported to the analysis institution for NGS analysis (Genomecare Inc, Seoul, Korea). Biopsy results were classified as euploidy, aneuploidy, mosaicism or no result.

Blastocyst vitrification, warming, and embryo transfer

Biopsied embryos were vitrified within 30 minutes using house solution. First, the embryos were vitrified with 1ml of HEPES medium (SAGE Quinn's-HEPES; CooperSurgical, Trumbull, CT, USA), 20% HSA (SAGE, CooperSurgical), 7.5% ethylene glycol (EG, E-9129; Sigma, St. Louis, MO, USA) and 7.5% using DMSO (Sigma-Aldrich, St. Louis, MO, USA) for 2.5 min. And then, it moved in same volume of 15% EG, 15% DMSO and 0.5M sucrose for 20 sec. The blastocysts were placed on an electron microscopic gold grid (EM Grid; SPI Supplies, West Chester, PA, USA) and immediately immersed into LN2. Embryo grid was put into the vial and stored at -196℃ using cane.

For warming, 4 step media were used, each containing 0.5M, 0.25M, 0.125M and 0.0M sucrose with HEPES medium and 20% HAS. The EM gold grid containing blastocysts was immersed in the first solution and then sequentially moved to the remaining solution at 2.5 minutes intervals. After thawing, blastocysts were washed in culture media and incubated overnight in a 37℃ incubator.

Frozen embryo transfers were performed as a single euploid blastocyst transfer cycle, with embryos biopsied on either day 5 or day 6. Clinical prerenancy rate was first evaluated as blood β-HCG 10~14 days after embryo transfer, and fetal heart movement was evaluated by ultrasound

between 6 and 8 weeks of pregnancy. Miscarriage rate was defined as the rate of intrauterine clinical pregnancies missed before 12 weeks of gestation.

Statistical analysis

Data analysis was performed using IBM Statistical Packages for Social Sciences (SPSS) version 23.0. The baseline data were compared using one way ANOVA and post-analysis. The chi-square test was used to compare categorical data. p values ≤ 0.05 were considered statistically significant.

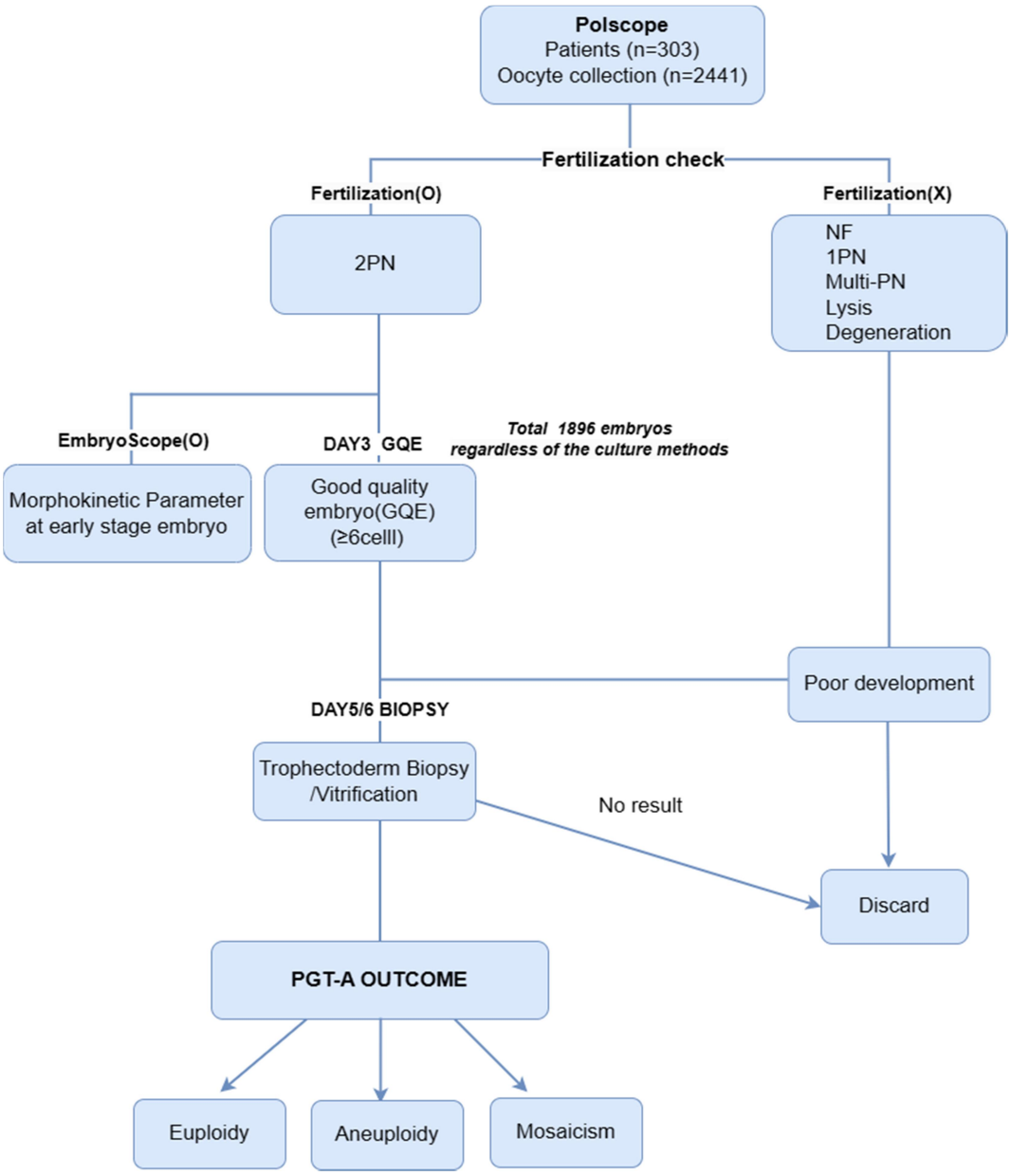

Figure 2. Process of study

NF, Non fertilized; 1PN, Monopronuclear; Multi-PN, Multi-pronuclear; Lysis, the disintegration by oocyte membrane rupture; Degeneration, characterized by lysis of the oocyte;

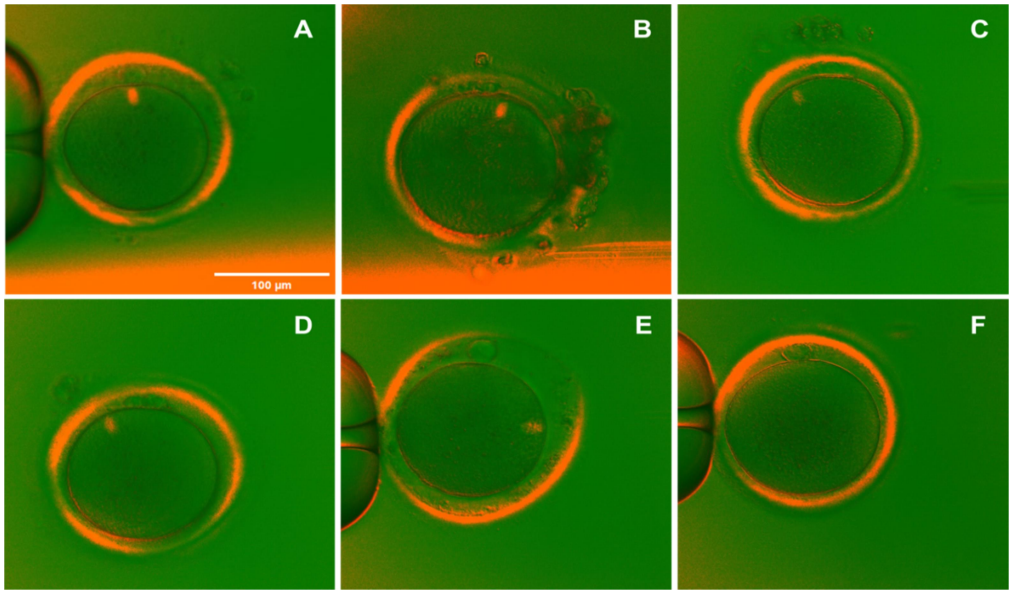

Figure 3. Oocyte meiotic spindle view with polscope

(A) Oocyte in group A1 having spindle 0° –5° beneath the polar body. (B) Oocyte in group A2 having spindle 5° –15° adjacent to the polar body. (C), (D), (E) Oocytes in group A3 having spindle ≥16° away from the polar body. (F) Oocyte in group A4 with no visible spindle.

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RESULTS

Description of patients

According to our results, a total of 303 patients had a mean length of infertility period of 2.6 ± 2.5 years with an average of 4.7 ± 3.3 IVF cycle failures. The mean patient's age was 39.8 ± 3.3 years. In addition, the groups consisted of following infertility factors: female factor, male factor, a history of repeated spontaneous abortion (RSA), a history of repeated implantation failure (RIF), genetic factor, unexplained causes, and a combination of two or more these factors. The reproductive characteristics of the patients can be seen in Table 1.

Table1. Reproductive characteristics of patients

Clinical parameter	Patient group (n = 303)
Duration of infertility (years)	2.6 ± 2.5
Average number of IVF treatments	4.7 ± 3.3
Age (years)	
Female	39.8 ± 3.3
Male	41.5 ± 4.6
BMI (kg/㎡)	21.7 ± 2.9
Basal FSH (IU/L)	7.3 ± 4.3
AMH (㎍/㎖)	2.5 ± 2.3
AFC	10.7 ± 5.8
Infertility factors(%)	
Female factor	35.3(107/303)
Male factor	27.7(84/303)
RSA	13.8(42/303)
RIF	9.9(30/303)
Genetic factor	6.9(21/303)
Unexplained	6.2(19/303)

BMI, body mass index; FSH, follicle stimulating hormone; AMH, anti-müllerian hormone; AFC, antral follicle count; RSA, repeated spontaneous abortion; RIF, repeated implantation failure

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Relationship between spindle angle, fertilization and development rates

A total of 2441 MII oocytes collected from 303 patients were classified depending on the angle position: group A1 (n = 947), A2 (n = 620), A3 (n = 584), and A4 with no visible spindle (n = 67). Of the most oocytes undergoing ICSI treatment, two pronuclei (2PN) formed 14–16 hours after the treatment. The non-fertilized (NF), abnormal fertilized (mono and multi PN), lysed, and degenerated oocytes were excluded. A flow diagram giving an overview of the samples is provided in Fig 1.

There was no significant difference in the fertilization rate among the groups with visible spindles (A1 vs. A2, p = 0.54; A2 vs. A3, p = 0.97; A1 vs. A3 group, p = 0.57). However, the non-visible spindle group, A4 showed a significantly lower fertilization rate than other groups (A1 vs. A4; p = 0.0005, A2 vs. A4; p = 0.0002, A3 vs. A4 group; p = 0.0003) (Fig. 3A).

In addition, the good quality embryo (GQE) rate on day 3 by spindle angle showed numerical values of A1 (86.8%), A2 (86.2%), A3 (83.6%), and A4 (77.8%). A1 and A2, near the PB, showed a significant difference from the non-visible A4 (A1 vs. A4; p = 0.0017, A2 vs. A4; p = 0.0066), while A3, away from the PB, did not (p = 0.074) (Fig. 3B).

Blastocysts obtained from each group were used for PGT-A biopsy. The utilized blastocyst rate was as follows: A1, 45.1%; A2, 44.2%; A3, 43.8% and A4, 28.8%. It should be noted that the blastocyst formation rate in A4 was significantly lower than the other groups (A1 vs. A4, p = 0.0005; A2 vs. A4, p = 0.0002; A3 vs. A4, p = 0.0002), while there was no significant difference among visible spindle group (p > 0.05) (Fig. 3C).

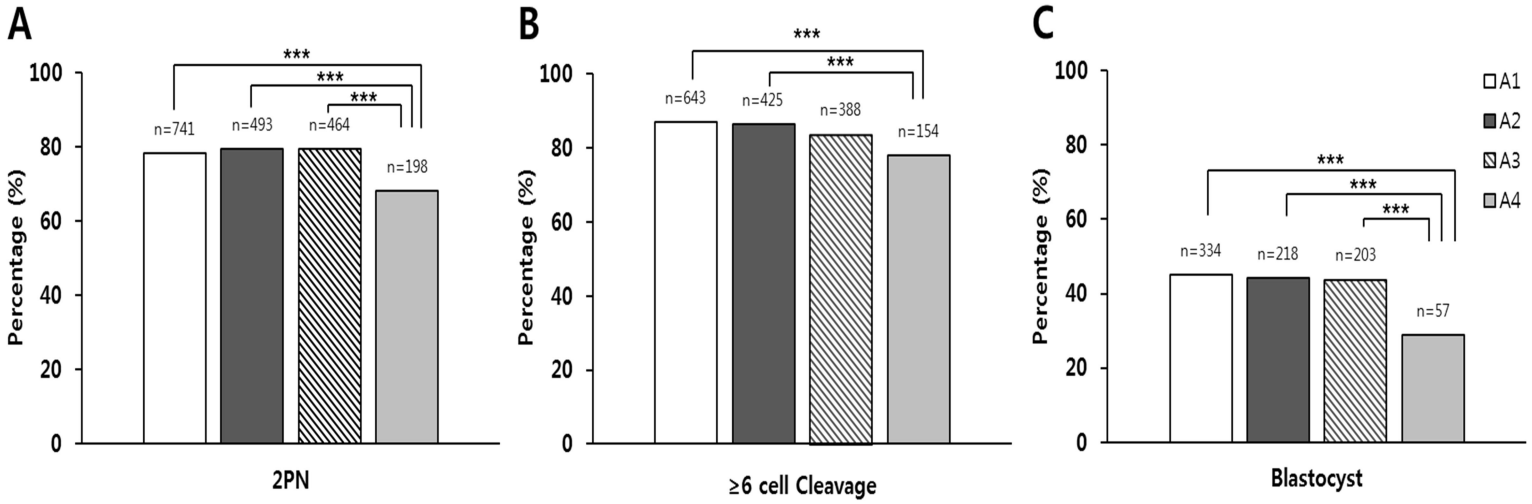

Figure 4. Relationship between spindle localization, oocyte competence and embryo development

The association between oocyte or embryo competence and spindle angle was observed with two pronuclei (2PN) status (A), good quality embryos (GQE) of 6 cells or more were examined on day 3 cleavage stage (B), and the blastocyst rate of trophectoderm (TE) biopsy on day 5 or 6 (C). The asterisks denote statistically significant differences when comparing values with each group: *** $P < 0.001$. Values are expressed as mean ± SEM.

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Comparison of euploid blastocyst rates in different spindle angles

The results showed that the spinple location related with oocyte maturation. Besides, the the oocyte meiotic spindle is associated with non-disjunction and chromosomal instability, we hypothesized that the localization of the oocyte meiotic spindle might affect the embryo euploidy in the human IVF cycle. As shown in Figure 4A, the whole patient group, regardless of age, the euploidy rate by spindle angle showed numerical values of A1 (n=59, 17.7%), A2 (n=46, 21.1%), A3 (n=38, 18.7%), and A4 (n=8, 14.0%). There was no significant difference in the euploidy rate by a spindle ($p > 0.05$).

Euploidy rates in the group of young patients (≤37 age) did not differ among groups, regardless of the localization of the spindle. The percentage of euploid blastocysts were 30.9% (n=29) in A1, 37.9% (n=22) in A2, 28.8% (n=17) in A3, and 18.8% (n=3) in A4 58.78%. The difference in euploidy rates between the four groups was insignificant ($p > 0.05$) (Fig. 4B).

In addition, the euploidy rates in the groups over 37 years of age, A1 (n=30, 12.6%), A2 (n=24, 15.1%), A3 (n=21, 14.7%), and A4 (n=5, 12.2%), also showed no significant difference regardless of the position of the spindle ($p > 0.05$) (Fig. 4C). Although there were no significant differences the results showed a possibility of the relationship between spindle angle of MII oocyte and chromosomal segregation.

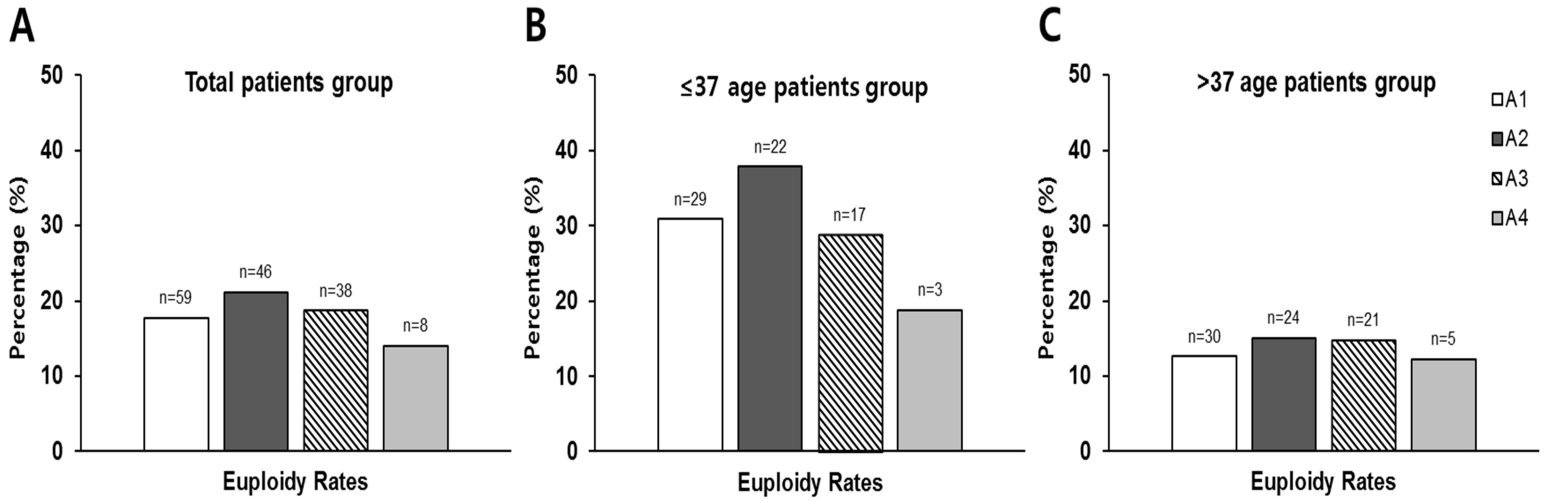

Figure 5. Comparison of euploid blastocyst rates in different spindle angles

The euploidy rates were analyzed after trophectoderm (TE) biopsy in in vitro fertilization (IVF) / preimplantation genetic testing for aneuploidy (PGT-A) cycles. The rates of euploid blastocyst were examined in groups of total patients (A), under 37 of age (B), or over 37 of age (C), respectively.

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Comparison of aneuploidy blastocyst rates in different spindle angles

The correlation between the relative spindle angle and aneuploidy rates were conformed in the human IVF cycle according to the patient's age. In the spindle group of A1, A2, A3, and A4, except for the age condition, the aneuploidy rates sequentially increased numerical values from A1 (n=197, 59.0%), A2 (n=129, 59.2%), A3 (n=129, 63.5%), to A4 (n=37, 64.9%) (Fig. 5A).

In the group under the age of 37, the aneuploidy rates by spindle angle showed numerical values A1 (n=31, 33.0%), A2 (n=20, 34.5%), A3 (n=20, 33.9%), and A4 (n=8, 50.0%) (Fig. 5B). Although there was no significant difference among each group, the aneuploidy rate in A4 was numerically higher than the other groups.

The aneuploidy rates in the groups over 37 years of age, A1(n=166, 69.5%), A2(n=109, 68.6%), A3(n=109, 76.2%), and A4(n=29, 70.7%), showed no significant difference regardless of the position of the spindle ($p > 0.05$) (Fig. 5C).

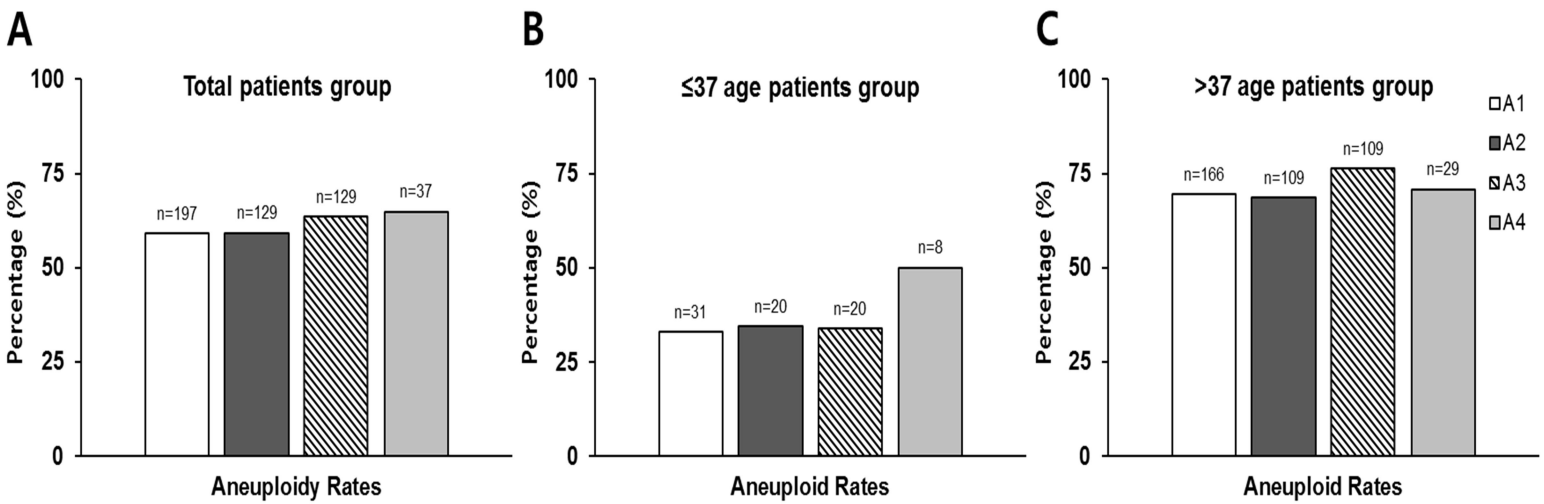

Figure 6. Comparison of aneuploid blastocyst rates in different spindle angles

The aneuploidy rates were obtained trophectoderm (TE) biopsy in in vitro fertilization (IVF) / preimplantation genetic testing for aneuploidy (PGT-A) cycles. The rates of aneuploid were examined in groups of total patients (A), under 37 of age (B), or over 37 of age (C), respectively.

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Correlation between morphokinetic parameters and PGT results for each spindle localization observed through timelapse

To investigate the effects of the relative spindle position on morphokinetic parameters of euploid, aneuploidy, and mosaic blastocysts, data collected from our patients undergoing PGT were assessed using the EmbryoScope™ time-lapse culturing system. The mean timepoints were compared for each spindle angle in euploid, aneuploidy, and mosaic blastocysts.

As shown in Table 2, there was a significant difference between spindle angles in tPNf and 2-cell embryo stage (t2) in the euploid embryo group. When post-analysis was performed, there was no significant difference when comparing each angle in tPNf. However, there was a significant difference between A1 (27.1 ± 3.0h) and A2 (25.2 ± 2.5h) mean values in t2. In the aneuploid embryo group, there was no significant difference among the four groups according to the morphokinetic parameters ($p > 0.05$) (Table 3).

The results of mosaic embryo groups showed significant differences when reaching the 7-cell embryo stage (t7) and 8-cell embryo stage (t8). Specifically, it showed significant differences between A1 (58.5 ± 9.1h) and A3 (52.6 ± 7.5h) at t7 ($p = 0.04$) and t8 between A1 (62.7 ± 10.7h) and A3 (56.0 ± 7.4h) ($p = 0.05$) (Table 4).

Table2. Mean of timepoints by spindle in the euploidy embryo groups

	A1 (n=130)		A2 (n=94)		A3 (n=84)		A4 (n=21)		
Variables	mean (hours)	(sd)	mean (hours)	(sd)	mean (hours)	(sd)	mean (hours)	(sd)	p-value
tPNf	24.6	2.9	23.1	2.5	24.2	2.1	25.6	3.4	0.05
t2	27.1	3.0	25.2	2.5	26.4	2.3	27.3	3.6	0.04 *
t3	37.5	4.7	34.8	3.5	37.4	3.7	37.7	2.6	0.12
t4	39.2	4.1	36.9	4.3	39.3	3.6	39.9	4.5	0.06
t5	49.2	7.1	47.6	5.7	51.4	5.4	51.6	6.0	0.12
t6	53.1	6.2	50.8	5.1	53.2	6.5	55.3	7.9	0.23
t7	56.4	7.2	53.3	6.0	57.1	7.9	56.8	9.0	0.19
t8	61.8	8.9	58.5	10.4	61.9	10.2	59.4	9.7	0.44
CC2(t3-t2)	10.2	3.6	9.8	2.9	11.3	1.4	10.8	0.2	0.35
CC3(t5-t3)	12.4	4.0	12.2	2.5	14.2	2.6	14.6	1.7	0.12
S1(t2-tPNf)	2.5	1.3	2.1	0.5	2.3	0.7	2.1	0.5	0.52
S2(t4-t3)	2.3	2.4	1.8	2.4	2.4	2.9	4.1	3.0	0.45
S3(t8-t5)	12.3	6.7	11.3	8.3	10.7	8.2	7.8	7.4	0.49

Post-analysis; t2 (A1 vs. A2; $p = 0.02$)

The one-way analysis of variance (ANOVA) was used for the statistical analysis.

Values are presented as means and SD. ***:** $p < 0.05$ indicates a significant difference.

Table3. Mean of timepoints by spindle in the aneuploidy embryo groups

	A1 (n=130)		A2 (n=94)		A3 (n=84)		A4 (n=21)		
Variables	mean (hours)	(sd)	mean (hours)	(sd)	mean (hours)	(sd)	mean (hours)	(sd)	p-value
tPNf	24.5	3.2	24.2	3.1	24.2	3.1	26.0	3.8	0.13
t2	27.0	3.6	26.8	3.4	27.0	3.6	28.1	4.2	0.51
t3	37.5	4.9	37.2	4.3	36.9	5.0	39.1	4.9	0.41
t4	39.2	4.9	39.3	4.8	39.1	4.6	40.6	4.1	0.68
t5	49.8	7.3	50.8	6.4	49.9	7.2	51.1	7.0	0.68
t6	53.8	6.7	53.4	6.9	53.1	6.6	55.2	5.4	0.65
t7	56.6	8.4	56.9	8.9	57.1	8.3	58.8	9.1	0.77
t8	61.1	9.7	61.5	10.8	61.7	9.0	63.0	11.4	0.86
CC2(t3-t2)	10.6	2.6	10.6	2.4	10.5	2.3	10.4	3.5	0.98
CC3(t5-t3)	12.6	4.4	13.3	4.3	13.1	4.7	12.0	5.5	0.64
S1(t2-tPNf)	2.6	1.5	2.6	1.3	2.8	1.6	2.1	0.7	0.24
S2(t4-t3)	2.2	2.4	2.2	2.9	2.4	2.8	2.4	2.8	0.67
S3(t8-t5)	11.3	7.9	10.8	8.5	11.6	8.4	12.2	9.7	0.91

The one-way analysis of variance (ANOVA) was used for the statistical analysis.

Values are presented as means and SD. ***:** $p < 0.05$ indicates a significant difference.

Table4. Mean of timepoints by spindle in the mosaicism embryo groups

	A1 (n=60)		A2 (n=31)		A3 (n=26)		A4 (n=9)		
Variables	mean (hours)	(sd)	mean (hours)	(sd)	mean (hours)	(sd)	mean (hours)	(sd)	p-value
tPNf	25.1	4.0	24.4	3.3	23.7	3.7	24.3	1.3	0.46
t2	27.6	4.1	27.3	4.1	26.0	3.5	26.9	1.0	0.36
t3	38.0	5.1	38.1	5.6	36.2	5.1	38.0	1.9	0.56
t4	40.1	5.4	40.0	5.1	37.5	4.2	39.4	2.3	0.19
t5	51.8	6.8	51.9	8.0	48.3	8.7	50.1	3.3	0.25
t6	54.7	7.2	53.6	8.4	51.3	7.9	52.6	2.3	0.31
t7	58.5	9.1	57.5	7.7	52.6	7.5	56.9	5.0	0.04 *
t8	62.7	10.7	61.5	10.1	56.0	7.4	59.9	7.3	0.04 *
CC2(t3-t2)	10.8	2.6	10.9	3.0	10.4	2.4	10.9	1.2	0.92
CC3(t5-t3)	13.1	4.6	12.9	6.5	13.9	4.0	12.1	3.7	0.88
S1(t2-tPNf)	2.5	1.4	2.9	2.6	2.2	0.9	2.6	0.8	0.54
S2(t4-t3)	2.4	3.2	2.0	2.1	1.6	1.9	1.6	1.2	0.55
S3(t8-t5)	11.3	8.8	11.7	8.1	7.5	6.0	10.3	8.0	0.24

Post-analysis; t7 (A1 vs. A3; $p = 0.04$), t8 (A1 vs. A3; $p = 0.05$)

The one-way analysis of variance (ANOVA) was used for the statistical analysis.

Values are presented as means and SD. ***:** $p < 0.05$ indicates a significant difference.

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Clinical outcomes based on spindle angles

A total of 84 cases of frozen-thawed cycles of euploidy embryos were performed, and each was divided by spindle position to examine the pregnancy and miscarriage rates. The clinical pregnancy rates were A1, 62.1%; A2, 64.5%; A3, 54.5%; and A4, 100% ($p > 0.05$). There was no miscarriage in A1 and A4 groups. The miscarriage rates in A2 and A3 groups were 10.0% and 8.3% ($p > 0.05$), respectively. Both clinical outcomes, pregnancy and miscarriage rates, were not significantly different depending on the position of the spindle (Table 5).

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Table 5. Percent of clinical outcomes based on spindle angle groups

	A1	A2	A3	A4	*P*-value
Clinical pregnancy rate (%)	62.1 (18/29)	64.5 (20/31)	54.5 (12/22)	100.0 (2/2)	$p > 0.05$
Miscarriage rate (%)	0.0 (0/12)	10.0 (2/20)	8.3 (1/12)	0.0 (0/2)	$p > 0.05$

The one-way analysis of variance (ANOVA) was used for the statistical analysis.

*: $p < 0.05$ indicates a significant difference.

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Discussion

Meoitic cycle regulation and maturation of oocyte is a key factor for normal fertilization and development. In human IVF various criteria have been employed to predict the oocyte maturation including the location and number of nucleoli, cytoplasmic colors, perivitelline spane, etc. Recently, with the high resolution machine the cellular oraganells are also used for criteria.

The significant findings of this study are as follows: (1) the fertilization rate in A4, the non-visible spindle group, was significantly lower than those in groups with a visible spindle; (2) Among groups with a spindle, only A1 and A2 showed significant differences in the GQE rate from the A4; (3) the groups having a spindle showed statistically higher utilized blastocyst rates of biopsy than those in the group without a spindle on day 5 or 6; (4) the aneuploidy rate in A4 was numerically lower than the other groups, and (5) the morphokinetic parameters were affected by the spindle position in euploidy and mosaicism groups. This study is the first report characterizing the association between the oocyte meiotic spindle angle based on PB and the blastocyst euploidy and evaluating the correlation between morphokinetic parameters and PGT results for spindle localization using the timelapse culture system.

The oocyte meiotic spindle is a microtubule structure and is associated with the success of chromosome separation (Coticchio et al., 2004). The chromosome segregation process is susceptible to the timing of spindle formation or even small changes in biochemistry (Wasielak et al., 2022). Therefore, factors affecting the meiotic spindle structure in the oocyte may be related to the ovarian stimulation protocol. It is also reported that defects

in the protein complex of the meiotic spindle, low energy supply, or disturbance of signal transduction pathways may be related to spindle structure (Eichenlaub et al., 2002). Moreover, because it stabilizes the spindle by temperature control, the stabilization of the spindle can be damaged by changes in temperature during oocyte collection, culture, pH of culture medium, or microinjection procedure (Wang et al., 2001; Cooke et al., 2003; Rienzi et al., 2003; Hu et al., 2001; Roberts et al., 2002).

Several studies reported the usefulness of spindle in human IVF.However, it is controversial. In some studies, oocytes with visible spindles have higher fertilization rates, embryo developmental competence, or even a higher proportion of blastocyst formation (Wang et al., 2001, Cohen et al., 2004, Rama Raju et al., 2007, Mahfoudh et al., 2017). However, a few other studies failed to demonstrate the same results, which did not observe any significant difference in fertilization rate, cleavage development rate, or implantation rates based on the presence of a spindle (Moon et al., 2003, Mahfoudh et al., 2017). The results of these studies have been contradictory.

In the present study, to evaluate the possibility of spindle in human IVF, the spindle angle to confirm the difference in the fertilization and development rates according to the angle. The results showed a significant difference in fertilization and embryonic development rates between oocytes with the visible spindle and the oocyte without the visible spindle. Additionally, the results demonstrated that fertilization and embryonic development rates were similar in the oocytes with spindles regardless of spindle position. It might be explained by avoiding visualized spindle in the oocyte, which minimizes the possibility of damaging the meiotic spindle that causes potential degeneration of the oocyte during ICSI. Although it is not

the first study to demonstrate the relationship between spindle, fertilization and embryo development, we conducted it with a larger patient group than other published studies. Thus, it showed more real significance compared with other studies.

In here, a visible spindle in the MII oocyte is a factor for prediction of improving developmental quality of early-stage embryo. In contrast, the relative position of the spindle within the oocyte does not appear to influence the developmental potential of embryos.

As mentioned above, the oocyte meiotic spindle is associated with non-disjunction, chromosomal instability, and developmental competence. Thus, we further demonstrated the association between aneuploidy and spindle, which may induce cell cycle arrest. In this study, the correlation between spindle angle and PGT-A results showed no significant difference in both euploidy and aneuploidy groups. However, the values of the aneuploidy rate of the entire patient group increased sequentially from A1, A2, and A3 to A4. It showed a tendency that A3, which has a spindle away from PB, and A4 may have a higher aneuploidy rate than A1 and A2. However, there is no significant difference in the aneuploidy rate because the ratio of spindles in A1 and A2 is numerically more remarkable than in A3 and A4. Significant results can be expected if more data is collected in further study.

In many studies, the advanced maternal age (AMA) is still controversial about how old each institution sets the standard, but this study was based on age 37. In women of advanced maternal age, the fertility rate rapidly decreases, and the spontaneous abortion rate increases rapidly (Heffner, 2004). These patients also have a high risk of early or late miscarriage or chromosomal abnormalities (Zhu et al., 2016). The other study suggests that

when AMA increases, the ratio of euploidy decreases, and there is a strong association between AMA and the incidence of aneuploidy (Liu et al., 2020). Based on the age of 37, the euploidy rate was not significantly different A1 (30.9% vs. 12.6%), A2 (37.9% vs. 15.1%), A3 (28.8 vs. 14.7%), A4 (18.8% vs. 12.2%), but it could be seen that it is numerically lower in all groups over 37. Therefore, it supports many studies that the euploidy rate is low in patients over 37 years of age so that embryos can be selected and transferred with PGT-A, and the live birth rate can be increased and the miscarriage rate decreased (Sato et al., 2019, Neal et al., 2018).

The time-lapse culture system, frequently handled by IVF labs, can improve reproductive outcomes of IVF treatment by providing less handling and more information by continuously culturing in a stable environment for developing non-invasive embryos. In addition, a morphokinetic parameter through timelapse can be used to estimate the viability and transfer of the embryo (Lemmen et al., 2008, Arav et al., 2008). These findings suggest that morphokinetic parameters can complement current embryo selection methods to increase the clinical pregnancy rate of IVF (Marcos et al., 2012).

In the PGT-A results through embryoscope culture, differences in timepoint for early-stage embryo development by spindle were also investigated. A significant difference was found between A1 (27.1 ± 3.0h) and A2 (25.2 ± 2.5h) in t2 of the euploid embryo group. The 2-cell division timepoint in euploidy was faster in A2 than in A1. In mosaicism, A1 and A3 showed significant differences at t7 and t8. The timing of division into 7-8 cells seems abnormally fast in A3, suggesting that the mosaicism rate can be increased. Lee et al. suggested that chromosomal abnormalities were divided into low and high in mosaicism embryos and compared with euploidy, and

high-level mosaic blastocysts were significantly delayed at t8 (Lee et al., 2019).

Further research on morphokinetic parameters between euploidy and aneuploidy is being actively conducted, but many opinions remain on the study's results. While some studies showed no significant difference by comparing the time points of euploidy and aneuploidy (Le et al., 2021, Zhang et al., 2017; Minasi et al., 2016), other studies showed significant differences but different time points (Campbell et al., 2013, Lee et al., 2019). In the present study, significant values between timepoints have been found, but this cannot yet be the standard for morphokinetic parameters. More data should be collected, and further research is needed.

The clinical outcome investigated the pregnancy rate and miscarriage rate when euploid embryos were transferred. There was no significant difference in pregnancy and miscarriage rate by the spindle. It was difficult to see the significance because the number of patients who thawed and transferred the euploid embryo was small, and the follow-up time was short to see the miscarriage rate. Some studies have shown higher pregnancy and birth rates when the spindle angle is close to the PB compared to non-visible spindles. However, there was no significant difference in the miscarriage rate (Mahfoudh et al., 2017). It is expected that more significant clinical outcomes will be obtained if the clinical results are continuously followed up.

In conclusion, MII oocytes in which the birefringent spindle can be visualized have a higher fertility rate and embryonic development competence than MII oocytes which are not observed meiotic spindle at the time points. In addition, the larger the oocyte meiotic angle, the higher the aneuploidy rate, indicating the tendency to increase the aneuploidy rate as

the spindle angle increases. Research has also been conducted through timelapse, a non-invasive evaluation that is currently widely used. Significance was also found at the timepoints of early embryo stage development by the angle of the euploidy and aneuploidy groups. Although this study did with limited number of oocytes and patients, this is the first study to reveal the relationship between the spindle angle and PGT-A, and later the spindle angle is a predictor for the blastocyst euploidy, which can help with embryo selection based on timepoints.

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논문개요

난자 내 방추사 위치에 따른 착상 전 유전진단에서 배수체와의 상관관계

난자의 성숙은 수정과 이후의 배 발생을 가능하게 하는 요인으로 인간의 체외 수정에서 가장 중요한 요인으로 알려져 있다. 난자의 성숙은 성공적인 감수분열을 포함하는데 감수분열 방추사의 교란은 염색체의 비분리 및 불안정성을 발생하며 배아의 발달능력에 영향을 미친다. 인위적으로 정자를 난자의 세포질로 주입할 때 감수분열 방추사의 교란을 피할 수 있는 방법은 편광현미경을 사용하여 시각화하는 것이다. 하지만, 난자의 방추체 형성의 특성을 비추어볼 때 난자의 감수분열 방추사들의 위치와 배아 배수체 사이의 관계는 아직 체외수정에서 잘 연구되지 않았다. 따라서 본 연구에서는 체외수정을 하는 환자에서 배아 발달에 대한 실시간 배아 발달 모니터링 시스템을 사용하여 최적의 배아를 선택하기 위한 형태동태학적 특성을 분석한 후 감수분열 방추사의 각도의 편차와 환자의 배 발달 및 배수체 사이의 연관성을 조사하였다. 본 연구는 2022년 3월부터 2023년 1월까지 편광현미경 관찰 및 미세수정한 후 PGT-A를 시행한 303명의 환자의 2441개 난자를 대상으로 진행하였다. 제1극체 위치에서 감수 분열 방추사 각도의 편차에 따라 4개의 군으로 나뉘었다: A1 (0-5^{o}), A2 (5-15^{o}), A3 (>15^{o}) 및 A4 (검경안됨). 또한, 배아는 난자 감수분열 방추사들의 각도에 따라 단일 배양하였고 배아 발달 능력을 평가하기 위해 실시가 배아 모니터링 시스템을 사용하여 모니터링하였다.

본 연구에서는 보이지 않는 방추사군 A4가 방추사를 가진 군들에 비해 수정률이 유의하게 낮은 것을 확인하였다 (A1 vs. A4; p = 0.0005, A2 vs. A4; p = 0.0002, A3 vs. A4; p = 0.0003). 방추사 각도의 편차에 따른 good quality

embryo (GQE) 비율은 A1 (86.8%), A2 (86.2%), A3 (83.6%), A4 (77.8%)의 수치를 보였다. 방추사들이 있는 그룹 중 A1과 A2만 보이지 않는 그룹 A4와 비교하여 GQE 비율에서 유의미한 차이를 보였다 (A1 vs. A4; p = 0.0017, A2 vs. A4; p = 0.0066). 또한, 5일 또는 6일째 생검 시 방추사들이 있는 그룹이 방추사들이 없는 그룹보다 통계적으로 더 높은 배반포 생검율을 보였다 (A1 vs. A4; p = 0.0005, A2 vs. A4; p = 0.0002, A3 vs. A4, p = 0.0002). 방추사에 의한 배수체 배아 비율에는 유의한 차이가 없었다 (p > 0.05). A1과 A2의 형태운동학적 매개변수 중 tPNf와 t2는 각 정배수체 그룹에서 A1과 A2를 비교하여 유의한 차이가 있었다. 모자이시즘 그룹에서는 A1과 A3를 비교하여 t7 (p = 0.04)과 t8 (p = 0.05)에서 유의한 차이를 보였다.

이 연구에서 난자 내 방추사의 위치는 수정과 배아발달, 배반포 발생률에서 유의적인 의미를 갖는 것을 확인하였다. 또한 난자 내 방추사의 위치에 따른 초기 배아 단계의 시점에서 morphokinetic parameter는 발달 가능성이 가장 높은 배수체 배아를 선별하는 데 필수적인 정보를 제공할 수 있을 것이라 말할 수 있다. 한편 각 그룹당 MII 난자의 수가가 매우 제한적이어서 통계적 의미성을 말하기 어려운 점이 있으나 향후 지속적 연구를 토하여 방추체의 위치와 염색체 수 이상의 관계를 이해함으로 활용 가능성을 확보할 수 있음에 그 의미가 크다 할 수 있다.

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2023 년 7 월

윤 솔 아