Assessment of developmental potential of human single pronucleated zygotes derived from conventional in vitro fertilization

Yoshiteru Kai¹ · Hitomi Moriwaki¹ · Keitaro Yumoto¹ · Kyoko Iwata¹ · Yasuyuki Mio¹

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Abstract

Purpose The aim of this study was to non-invasively validate the developmental potential of human single pronucleated (1PN) zygotes derived from conventional in vitro fertilization (c-IVF) at the zygote stage.

Methods Fifty 1PN zygotes derived from 45 patients undergoing c-IVF were used. Immunohistochemistry and fluorescence live cell imaging were used to confirm normal chromosome segregation during the first mitosis. The usefulness of measuring pronuclear diameter was assessed on the basis of the presence or absence of a proper first cleavage and validated by subsequent development.

Results Although approximately 80% (15/19) of 1PN zygotes contained a diploid genome, immunohistochemistry revealed an unequal distribution of paternal and maternal genomes at the first mitosis. Fluorescence live imaging revealed that 73% (8/11) of 1PN zygotes formed a functional mitotic spindle at the first mitosis resulting from diploid genomes, with 25% (2/8) of these forming a tripolar spindle. 1PN zygotes in which the pronucleus disappeared and that subsequently underwent cleavage had a pronuclear diameter $\geq 32.2 \mu\text{m}$. The selection of 1PN zygotes based on pronuclear diameter resulted in zygotes that all formed mitotic spindles with poles during cleavage. Furthermore, 63% (5/8) of these zygotes reached the blastocyst stage.

Conclusions This study demonstrates the usefulness of a non-invasive assessment of 1PN zygotes derived from c-IVF as an indicator of developmental potential. Furthermore, diploid 1PN zygotes do not always exhibit normal chromosome segregation at the first mitosis. A pronuclear diameter $\geq 32.2 \mu\text{m}$ just before PN breakdown might be a useful criterion to assess 1PN zygotes that are capable of further development.

Keywords Single pronucleus (1PN) · Mitotic spindle · Chromosome segregation · Fluorescence live cell imaging

Abbreviations

5mC	5-Methylcytosine
5hmC	5-Hydroxymethylcytosine
H3K9me3	Histone H3 trimethyl lysine 9
MAP4	Microtubule associated protein 4
PN	Pronucleus (plural: pronuclei)/pronuclear
PB	Polar body
c-IVF	Conventional in vitro fertilization
ICSI	Intracytoplasmic sperm injection
PGS	Preimplantation genetic screening

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✉ Yasuyuki Mio
yasmio@mfc.or.jp

¹ Fertility Research Centre, Mio Fertility Clinic, 2-1-1 Kuzumo-Minami, Yonago 683-0008, Japan

Introduction

In assisted reproductive technologies, normal fertilization is attained on the formation of two pronuclei (2PN) and the extrusion of the second polar body (PB) into the perivitelline space. However, zygotes with a single pronucleus (1PN) are often encountered in the clinical setting. Previous studies have reported that there are several possible fertilization patterns for human 1PN zygotes, such as parthenogenesis, incorporation of both male and female pronuclei, asynchrony in pronucleus formation, and extrusion of a third PB-like material [1–5].

According to cytogenetic analyses, only approximately 30% of 1PN zygotes derived from intracytoplasmic sperm injection (ICSI) are diploid [6–8]. These findings suggest that the majority of 1PN zygotes derived from ICSI are generated by parthenogenesis. Conversely, cytogenetic analyses identify higher rates (46–80%) of 1PN zygotes obtained from conventional in vitro fertilization (c-IVF) [2, 9]. Genome composition analysis based on histone modification has revealed that 87%

of 1PN zygotes derived from c-IVF contain a diploid genome [7]. In addition, based on analyses of DNA methylation status and centrosome numbers, it has been reported that approximately half the 1PN zygotes derived from c-IVF contain a diploid genome [10]. Furthermore, it has been reported that approximately 45% of 1PN zygotes derived from c-IVF show signs of sperm penetration [1]. Indeed, some 1PN zygotes derived from c-IVF are able to develop to the blastocyst stage, and healthy babies have been born after embryo transfer using 1PN zygotes [11, 12]. Based on these observations, some 1PN zygotes may have the potential to develop into normal babies. However, it is also known that 1PN zygotes generated by parthenogenesis can still develop to the blastocyst stage [13, 14]. Therefore, we need to establish a scientifically valid screening method to select usable 1PN zygotes for clinical applications. Although, as mentioned above, the existence of usable diploid 1PN zygotes derived from c-IVF has been reported, the characteristics of these zygotes are unknown. Preimplantation genetic screening (PGS) of blastocysts can support the diagnosis of a diploid genome, but a prompt and non-invasive method is better for embryos.

In the present study, we focused on the first mitosis to assess the developmental potential of 1PN zygotes derived from c-IVF. Furthermore, we propose advanced criteria based on the diameter of the pronucleus to identify diploid 1PN zygotes with developmental potential.

Materials and methods

Collection of zygotes

Zygotes were donated from infertile couples undergoing c-IVF cycles. Zygotes with a single PB were excluded from the study. After syngamy assessment (more than 22 h post-insemination [h.p.i.]), 1PN zygotes were selected and used in the study (Table 1). Zygotes were frozen and thawed using the Cryotop methodology for human embryo vitrification (Kitazato).

Primary antibodies

The present study used mouse monoclonal antibodies against 5-methylcytosine (5mC; 1:100; Calbiochem) and α -tubulin (1:100; Abcam), as well as rabbit polyclonal antibodies against 5-hydroxymethylcytosine (5hmC; 1:100; Active Motif) and histone H3 trimethyl lysine 9 (H3K9me3; 1:300; Abcam).

Fixation and immunofluorescence

For imaging of DNA and histone methylation status, zygotes were fixed overnight at 4 °C in phosphate-buffered saline

(PBS) containing 2% paraformaldehyde, 0.2% Triton X-100, and 0.2% NI Protein Substitute (NPS; NAKA Medical). After washing with 0.2% NPS in PBS, the fixed specimens were denatured with 2 M HCl containing 0.2% NPS for 1 h before being incubated overnight at 4 °C with primary antibodies. For imaging of the mitotic spindle, zygotes were fixed overnight at 4 °C in microtubule stabilization buffer extraction fixative, consisting of 2% formaldehyde, 0.1 M piperazine-1,4-bis-2-ethanesulfonic acid (PIPES), 50% deuterium oxide, 0.01% aprotinin, 1 mM taxol, and 0.5% Triton X-100 [15]. After washing with 0.05% Tween 20 in PBS, the fixed specimens were blocked in 3% bovine serum albumin and 0.05% Tween 20 in PBS for 1 h and then incubated overnight at 4 °C with primary antibodies. Primary antibodies were detected using Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit IgG (Life Technologies). Images were obtained at 0.5- μ m intervals in the *z*-axis using an FV1000 confocal microscope (Olympus). The imaging region of the *z*-axis was set to a range that covered all the fluorescent signals.

Plasmids

The plasmid vectors used in the present study were pTagGFP2-H2B and pFusionRed-MAP4 (Evrogen). T7 promoter sequences were inserted anterior to the open reading frame for transcription into mRNA *in vitro*.

Fluorescence live cell imaging and time-lapse cinematography

Zygotes were placed in a CV1000 system (Yokogawa Electric), and images were captured at 37 °C in 6% CO₂ in air. Measurements of pronuclear diameter and time course calculations from disappearance of the pronucleus to first cleavage were made using bundled software. For fluorescent live cell imaging analysis, a mixture of TagGFP2-H2B mRNA and FusionRed-MAP4 mRNA was injected into zygotes using a micromanipulator (Narishige Group). The mRNA was transcribed *in vitro* by an mMESSAGE mMACHINE T7 Ultra Transcription Kit (Life Technologies). Fluorescent images were captured at 6-min and 1.2- μ m intervals in the *z*-axis over a range that captured all the fluorescent signals. Images of time-lapse cinematography without mRNA probes were captured at 20-min intervals. Blastocysts were evaluated using the Gardner grading system [16].

Statistical analysis

P values were calculated using an unpaired *t* test and two-sided *P* < 0.05 was considered significant. Pronuclear diameter data are presented as boxplots, with the median, interquartile range, and maximum and minimum values shown. In the text, data are presented as the mean $\pm$ SD where appropriate.

Table 1 Characteristics of the single pronucleated zygotes used in the present study

	2 PBs		3 PBs		Fragmented PB		Total
	F/T	Fresh	F/T	Fresh	F/T	Fresh	
5mC and 5hmC staining	5	—	—	—	5	—	10
H3K9me3 staining	—	4	1	—	4	—	9
α -tubulin and H3K9me3 staining	2	2	—	—	1	2	7
H2B and MAP4 live imaging	—	3	—	1	2	5	11
Group 1	—	(2)	—	—	—	(4)	—
Group 2	—	—	(1)	—	(2)	—	—
Group 3	3	—	—	—	2	—	5
Time-lapse cinematography (pronuclear diameter assessment)	2	—	—	—	3	—	5
H2B and MAP4 live-imaging (pronuclear diameter assessment)	—	2	—	—	—	1	3
Total	12	10	1	1	16	8	50

The numbers in parentheses represent the sample numbers also used in live-imaging

PB polar body, F/T frozen and thawed, 5mC 5-methylcytosine, 5hmC 5-hydroxymethylcytosine, H3K9me3 histone H3 trimethyl lysine 9, MAP4 microtubule associated protein 4

Results

Pronuclear genome composition according to DNA and histone methylation status

First, we reconfirmed genome composition on the basis of the DNA and histone methylation status in pronuclei of 1PN zygotes according to the previous study [10]. Eight of 10 zygotes (80%) showed a mixed 5mC-/5hmC-positive genome in the single pronucleus (Online Resource 1a). These zygotes probably contained both the maternal and paternal genomes. The remaining two zygotes showed a single pronucleus that was strongly positive for 5mC ($n = 1$) or 5hmC ($n = 1$) (Online Resource 1b, c). These results indicate that these two zygotes were produced by haploid parthenogenesis.

H3K9me3 immunofluorescence was evaluated in nine 1PN zygotes because H3K9me3 is specifically enriched in the maternal pronucleus not only in mice but also in humans [7, 17]. Seven of these nine zygotes (78%) had a non-uniform H3K9me3-positive pronucleus (Fig. 1a). These results imply that both paternal and maternal genomes were present in the zygotes analyzed, indicating that approximately 80% of 1PN zygotes in c-IVF procedures contain a diploid genome, a slight increase from previous results [10].

First mitotic spindle formation and chromosome segregation

To determine whether the paternal and maternal genomes were equally distributed during the first cleavage, α -tubulin and H3K9me3 immunofluorescence was evaluated at prometaphase, metaphase, anaphase, and telophase in seven 1PN zygotes. Both H3K9me3-positive and H3K9me3-

negative genomes were detected in three of four zygotes at prometaphase and metaphase (Fig. 1b, c). Although equal distribution of H3K9me3-positive (maternal) and H3K9me3-negative (paternal) genomes was detected in one zygote at anaphase, another zygote showed an unequal distribution of paternal and maternal genomes at telophase (Fig. 1d, e).

Next, fluorescence live cell imaging was performed to visualize histone H2B and microtubule associated protein 4 (MAP4) in order to evaluate whether the first mitotic spindles of 1PN zygotes are functional. The dynamics of the genome were visualized using a TagGFP2-H2B mRNA probe, and microtubules were visualized using a FusionRed-MAP4 mRNA probe. Although six of 11 zygotes showed bipolar spindle formation and cleaved to two cells (Fig. 2a; Online Resource 2), two zygotes formed a tripolar spindle, leading to aberrant chromosome segregation and first cleavage (Fig. 2b; Online Resource 3). The remaining three zygotes showed chromosome condensation, but spindles were not formed (Fig. 2c; Online Resource 4).

Classification of 1PN zygotes based on morphological assessment

To investigate whether morphological assessment can be used to classify zygotes, we retrospectively measured the diameter of single pronuclei using fluorescence live cell imaging data. Time-lapse data without mRNA probes ($n = 5$) were used in addition to fluorescence live cell imaging data for this analysis. The 1PN zygotes were divided into three groups: group 1, in which the pronucleus disappeared and embryos cleaved ($n = 6$); group 2, in which the pronucleus disappeared only ($n = 3$); and group 3, in which the pronucleus did not disappear and there was no cleavage ($n = 5$). Group 1 zygotes probably contained

Fig. 1 Immunohistochemical analysis of single pronucleated (1PN) zygotes. Genome distributions in 1PN zygotes during the first cleavage are shown by immunofluorescence staining of histone H3 trimethyl lysine 9 (H3K9me3; red) and α -tubulin (green). DNA is counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue). **a** Representative images of pronuclei stained with H3K9me3. H3K9me3 signals are detected across a widespread area, but parts of the pronucleus are negative for H3K9me3. **b, c** Images showing the retention of a diploid

genome at prometaphase and metaphase. Both H3K9me3-positive (maternal) and H3K9me3-negative (paternal) genomes are detected. **d** Images showing even genome distribution at anaphase. A mixture of H3K9me3-positive and H3K9me3-negative genomes is distributed to the polar microtubules, indicating the axis of cell division. **e** Images showing uneven genome distribution at telophase. The maternal and paternal genomes are arranged unequally around the axis of cell division. Scale bars = 10 μ m

diploid genomes because of spindle formation. The horizontal width of the pronucleus was measured as a diameter (Fig. 3a), and the diameter of the pronuclei in groups 1 and 2 was measured just before syngamy. The mean diameter of a pronucleus in group 1 was larger than that in group 3 (32.2 ± 3.1 vs. 27.4 ± 3.4 μ m, $P = 0.036$; Fig. 3b). There was no statistical difference between group 1 and group 2 (32.2 ± 3.1 vs. 30.3 ± 1.5 μ m, $P = 0.37$; Fig. 3b). The time-course from disappearance of the pronucleus to the first cleavage in group 1 was 2.5 ± 0.7 h (Fig. 3c).

Pronuclear diameter as a marker of developmental potential of 1PN zygotes

To determine whether assessment of pronuclear diameter was useful, the developmental potential of 1PN zygotes with a pronuclear diameter ≥ 32.2 μ m was analyzed prospectively. First, time-lapse cinematography analysis without mRNA probes was used on five 1PN zygotes (mean pronuclear diameter 34.0 ± 1.4 μ m) to determine whether these zygotes developed to the blastocyst stage. Four of these five zygotes reached the

blastocyst stage. Of these, three blastocysts were classified as being of good quality (i.e., quality 3BB or greater according to the Gardner grading system). Next, fluorescence live cell imaging was used to analyze the mitotic spindles during development using three 1PN zygotes (mean pronuclear diameter 34.0 ± 1.7 μ m). All three zygotes formed mitotic spindles with a pole at least during the third cleavage, which indicates that these zygotes contain centrosomes derived from spermatozoa. These three zygotes developed to the eight-cell, morula or blastocyst stage. In the case of the zygote that developed to the blastocyst stage, normal chromosome segregation was confirmed at the first and second mitosis, but one blastomere exhibited abnormal chromosome segregation in three different directions in the third mitosis (Fig. 4; Online Resource 5).

Discussion

Since 1978, many babies have been born from in vitro--produced human embryos [18]. Normally fertilized zygotes

Fig. 2 Fluorescence live cell imaging during the first cleavage showing histone H2B (green) and microtubule associated protein 4 (MAP4; red) during the first cleavage in single pronucleated zygotes. **a** Still images showing the formation of a bipolar spindle and even chromosome segregation. After disappearance of the pronucleus, the bipolar spindle is formed at metaphase. Even chromosome segregation is detected at anaphase. **b** Still images showing the formation of a tripolar spindle.

This zygote formed a tripolar spindle at metaphase, leading to uneven chromosome segregation at anaphase. **c** Still images showing disordered spindle formation. Even though chromosomes are condensed, the spindle is not formed and abnormal chromosome segregation occurs. Fluorescent images are full projections of *z* sections. The spindle pole is indicated by arrowheads. Scale bars = 20 μ m

exhibit characteristic morphology, with two PBs and two juxtaposed pronuclei. Abnormally fertilized human zygotes with one pronucleus have been identified in previous studies, with several causes proposed for the creation of 1PN zygotes, such as asynchronous pronucleus formation, pronuclear fusion, parthenogenesis, and extrusion of a third PB-like material [1–5].

According to the Istanbul consensus [19], fertilization is usually assessed 16–18 h after insemination and syngamy is assessed at 22–24 h.p.i. Asynchrony in pronucleus formation carries the risk of misjudging the zygotes to be 1PN zygotes even though they are normal. Therefore, it may be important to wait until assessment of syngamy before making a final judgment in the case of 1PN zygotes. In Mio Fertility clinic, we usually assess fertilization 16–17 h.p.i. Of the fertilized oocytes derived from c-IVF in Mio Fertility clinic in 2016, approximately 1% (192/19906) developed into 1PN zygotes.

According to studies reporting pronuclear fusion, it is possible that one nuclear envelope containing paternal and maternal genomes forms a single diploid pronucleus [4, 12]. These 1PN zygotes are thought to have the potential to develop into blastocysts. Recently, it was reported that 1PN zygotes derived from c-IVF can give rise to normal healthy babies using assisted reproductive technology [12]. Although these 1PN zygotes are thought to contain a diploid genome, pronuclear fusion has not been observed in an investigation using time-lapse cinematography from the time of the appearance of the male and female pronucleus until syngamy [20]. Thus, it is

more likely that the fusion of paternal and maternal genomes occurs before pronuclear formation. Although it remains a matter of speculation, irregular membrane formation surrounding maternal and paternal genomes can cause a single diploid pronucleus [21].

Conversely, 1PN zygotes caused by parthenogenetic activation (i.e., oocytes are stimulated and develop without the male genome) contain a haploid genome [4]. In addition, previous studies have revealed that the rate of development to the blastocyst stage is lower for 1PN zygotes derived from ICSI than c-IVF [8, 22, 23]. This is because the ICSI-derived 1PN zygotes arise primarily from parthenogenetic activation [24]. Furthermore, time-lapse cinematography study has revealed a novel phenomenon in which a third PB-like material was extruded into the perivitelline space shortly after extrusion of the second PB; these oocytes developed into 1PN zygotes and poor-quality embryos [5].

DNA methylation marks are actively removed from the paternal genome at the zygote stage [25, 26]. It is also known that the paternal pronucleus exhibits higher levels of hyperacetylated histone H4 than the female pronucleus [27]. Thus, the separation of maternal and paternal genomes by each pronuclear envelope is important in establishing differential epigenetic regulation. Paternal-specific DNA demethylation converting 5mC to 5hmC is induced by ten–eleven translocation 3 shortly after fertilization [28–30]. This conversion occurs selectively in the male pronucleus before the first

Fig. 3 Classification of diploid single pronucleated (1PN) zygotes based on measurements of the diameter of pronuclei and time course analysis. **a** Confocal bright field images of a 1PN zygote. The horizontal width of the pronucleus is measured as the diameter. Scale bar = 50 μm . **b** Boxplots comparing the pronuclear diameter of zygotes in group 1, group 2, and group 3, defined as follows: group 1, pronucleus disappeared and embryos cleaved; group 2, pronucleus disappeared only; and group 3, pronucleus did not disappear or there was no cleavage. **c** Boxplot showing the period from disappearance of the pronucleus to first cleavage in group 1 zygotes. The boxes show the interquartile range, with the median value indicated by the horizontal line; whiskers show the range

cell division in zygotes [31]. Previously, it has been reported that the mixed 5mC-/5hmC-positive single pronucleus results from the fusion of paternal and maternal genomes [10]. However, we need to carefully consider the possibility that conversion from 5mC to 5hmC occurs not only in the paternal genome but also in the maternal genome. Although paternal

and maternal genomes are separated by the pronuclear envelope of each pronucleus in normal 2PN zygotes, both genomes in a diploid 1PN zygote exist under the same conditions in a single pronucleus, together with ten–eleven translocation 3; therefore, these zygotes may not have a normal diploid status. However, we have also analyzed the H3K9me3 status of

Fig. 4 Mitotic spindles of single pronucleated (1PN) zygotes with a pronuclear diameter $\geq 32.2 \mu\text{m}$. Live-imaging analysis visualizing histone H2B (green) and microtubule associated protein 4 (MAP4; red) from the first to the third mitosis. The first and second mitotic spindles segregated chromosomes equally, leading to normal cytokinesis. At the third mitosis, one mitotic spindle in blastomere V divided the chromosomes in three different directions, whereas the other three spindles divided chromosomes to two to opposite poles

mitotic chromosomes at the first mitosis. H3K9me3 is enriched specifically in the maternal pronucleus not only in mice but also in humans [7, 17]. Immunofluorescence analysis for H3K9me3 revealed that 87% of human 1PN zygotes derived from c-IVF contain a diploid genome [7]. In the present study, we also detected non-uniform staining for H3K9me3 in 1PN zygotes using immunofluorescence. Furthermore, we found both H3K9me3-positive and H3K9me3-negative chromosomes at syngamy, indicating that the single pronucleus contained both paternal and maternal genomes.

Human oocytes lack centriolar structures, whereas human spermatozoa have centrioles; consequently, the zygotic centrosome of the human zygote must be derived from the spermatozoa [32]. Transmission electron microscopy revealed that centrosomes are duplicated at the pronuclear stage, when male and female pronuclei are in close association [32, 33]. Recently, immunofluorescent analysis has revealed that the number of centrosomes could be used to determine the number of spermatozoa incorporated into the zygote [10]. According to this theory, zygotes incorporating a spermatozoon must be able to form a bipolar spindle during mitosis. It has been reported that approximately half the 1PN zygotes can form a bipolar spindle during the first cleavage, but parthenogenetic 1PN zygotes cannot form a spindle because they lack centrosomes [34]. However, live-imaging analysis and immunofluorescence of the mitotic apparatus in the present study revealed that 1PN zygotes forming a spindle did not always exhibit normal chromosome segregation during the first cleavage. That is, not all diploid 1PN zygotes can distribute chromosomes normally along spindles during the first mitosis. This is probably because of the incomplete genome and/or centrosome function of a 1PN zygote. Oocyte immaturity may also contribute to spindle formation and function. In vitro maturation of oocytes increases the incidence of 1PN zygotes and abnormal cleavage rates [35]. The separate formation of male and female pronuclei is the accepted normal fertilization modality, and 1PN zygotes have not undergone the normal process, even if they have a diploid genome.

Time-lapse video cinematography analysis of human embryo development has revealed that the female pronucleus is slightly smaller than the male pronucleus [20]. Using time-lapse cinematography, we have found that the mean diameter of the male pronucleus is slightly larger than that of the female pronucleus (28.0 ± 2.1 vs. 26.4 ± 2.0 μm , respectively; unpubl. data). Therefore, in theory, a diploid 1PN zygote should have an approximate mean pronuclear diameter of 34.3 μm to ensure that there is enough space for the same amount of intrapronuclear structures as in a normal fertilized zygote. Indeed, chromosomal constitution analysis has revealed that 1PN zygotes with a pronuclear diameter ≥ 29 μm result mostly in diploid embryos [11]. Previous time-lapse study has shown that the average period from syngamy to the first cleavage is approximately 2.5 h in normal fertilized 2PN zygotes [36]. In

the present study, 1PN zygotes in which the pronucleus disappeared and first cleavage occurred had a mean pronuclear diameter of 32.2 ± 3.1 μm . Furthermore, the time from syngamy to the first cleavage was 2.5 ± 0.7 h, the same as for normal fertilized zygotes. It seems that these criteria (i.e., pronuclear diameter and time from syngamy to first cleavage) may be useful for determining diploid 1PN zygotes. Indeed, in the present study, we confirmed that the formation of mitotic spindles and normal development occurred at a high frequency in 1PN zygotes with a pronuclear diameter ≥ 32.2 μm . This indicates that a strategy focusing on the first mitosis is reasonable for the assessment of the potential for further embryonic development. Although the present study offers novel criteria to select 1PN zygotes with developmental potential at the zygote stage, they are not exclusive criteria. Currently, PGS of blastocysts derived from 1PN zygotes, such as chromosome karyotype analyses or SNP array, is the best method for the selection of embryos in the clinical setting.

In conclusion, on the basis of the presence or absence of a proper first cleavage, we have confirmed that measurement of pronuclear diameter can be used to select diploid 1PN zygotes derived from c-IVF. We hope that the findings of the present study help establish a screening method for the selection of usable 1PN zygotes for clinical applications and improve our understanding of the biological characteristics of 1PN zygotes.

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Author contributions Y.K. designed this research, performed experiments, analyzed the data, and wrote the manuscript. H.M. also analyzed the data and wrote the manuscript. K.Y. and K.I. were involved in the clinical zygote vitrification. Y.M. supervised this research.

Compliance with ethical standards

Ethics approval This study was approved by the Ethics Committee of JISART (Japanese Institution for Standardizing Assisted Reproductive Technology) and was registered with the Japan Society of Obstetrics and Gynecology.

Conflict of interest The authors declare that they have no conflict of interest.

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