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Potential of zygotes to produce live births can be identified by the size of the male and female pronuclei just before their membranes break down

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Abstract

Aim: To determine whether there are differences in size between the male and female pronuclei immediately before the pronuclear membrane breakdown (PNMBD) and to evaluate whether pronuclear size differences influence normal birth rates.

Methods: Time-lapse photography was used to measure the size of each pronucleus, while the outcome of 71 frozen-thawed single blastocyst transfers in patients receiving hormone therapy was analyzed retrospectively. The pronuclear measurements were made 4 hours before the PNMBD, corresponding to 16-20 hours after insemination or intracytoplasmic sperm injection, and immediately before the PNMBD. The differences in the areas between the pronuclei in the zygotes that were associated with the live births were compared with those that were associated with the failed pregnancies.

Results: The average difference in the area between the pronuclei 4 hours before and immediately before the PNMBD in the patients with a live birth was significantly smaller than in the patients with a failed birth. In addition, the average area difference in the patients with a successful birth was significantly smaller when the measurements were made immediately before the PNMBD, compared with the measurements 4 hours before the PNMBD. Such differences were not detected among the patients who did not achieve a birth.

Conclusion: The birth of healthy babies resulted from zygotes that contained pronuclei of similar size when the measurements were made immediately before the PNMBD. Evaluating the size of each pronucleus immediately before the PNMBD provides an effective indicator of the embryo's potential at an early stage of development.

KEYWORDS
frozen-thawed blastocyst transfer, live birth rate, pronuclear membrane breakdown, **p**ronuclear size, time-lapse observation

1 | INTRODUCTION

The size of the male pronuclei generally is known to be larger than that of the female pronuclei in the fertilized oocytes of mice. However, previous studies have reported that the size of human male and female pronuclei are almost equal.[1-3] Zygotes containing two pronuclei, in which the diameters differed by at least four microns, were found to arrest at a significantly higher rate and to have a significantly higher incidence of mosaicism than zygotes with pronuclear diameters that differed by less than four microns.[4] Furthermore, two studies reported that zygotes with different pronuclear sizes lead to a significantly higher incidence of both embryo cleavage arrest and mosaicism in day 3 embryos than do zygotes with pronuclei of a similar size.[4,5] Following these findings, two studies reported that the blastocyst development rates were significantly higher in the zygotes with two pronuclear areas of similar size than in the zygotes that contained pronuclei of unequal size.[6,7] In addition, another study reported that an unequal pronuclear size was correlated with a statistically significant slower cleavage rate and inferior embryo quality rate.[8] It was also reported that there was a higher incidence of chromosomal abnormalities in the embryos that originated from zygotes that contained pronuclei of different sizes.[9] However, pronuclear size evaluations are not very accurate and the difference in diameter sizes by themselves cannot predict chromosomal abnormality.

Fertilization is usually confirmed with the presence of male and female pronuclei 16–18 h after insemination or intracytoplasmic sperm injection (ICSI). More recently, the application of time-lapse observations has become available and regular measurements have been made in order to obtain more accurate information as both pronuclei continue to grow until the pronuclear membrane breakdown (PNMBD) occurs. Such more accurate measurements were used in the present study. The aim was to analyze the differences in size between the male and the female pronuclei immediately before the PNMBD and to investigate the relationship between the size differences and the potential for the birth of healthy babies.

2 | MATERIALS AND METHODS

A retrospective cohort study was used involving 71 frozen-thawed single blastocyst transfers (44 in vitro fertilization [IVF] and 27 ICSI cycles from 61 patients), in which the pronuclei of the zygotes were observed by a time-lapse system (EmbryoScope®; Vitrolife, Tokyo, Japan) from June 2013 to December 2014. The time-lapse data were reused from the authors' previously published study.[10]

2.1 | In vitro fertilization and intracytoplasmic sperm injection procedures

In the oocyte retrieval cycle, the patients were stimulated by using standard gonadotropin-releasing hormone agonist/follicle-stimulating hormone (FSH) protocols or the antagonist/FSH protocol. Ovulation induction was triggered when the second leading follicle was >18 mm in diameter. Ultrasound-guided transvaginal oocyte retrieval was performed 35-36 hours later.

The IVF laboratory procedures were as follows. Immediately on retrieval, the oocytes were placed in Universal IVF Medium (Origio a/s, Jyllinge, Denmark) that was overlaid with mineral oil (Irvine Scientific, Santa Ana, CA, USA). The oocytes were inseminated 3-5 hours later by using conventional insemination procedures or ICSI, depending on the semen parameters. After a fertilization check, performed ~18 hours after insemination, the resultant zygotes were placed in a global medium (LifeGlobal, Guelph, ON, Canada) that contained 10% HSA (LifeGlobal), in which they remained until day 5. One-to-four embryos were cultured in groups in a 50 μL droplet of culture medium under mineral oil.

The authors performed a stimulation of endometrium embryo transfer for most of the patients (~90%) with a single blastocyst transfer 3 days before a frozen-thawed blastocyst was transferred.[11] The embryo culture supernatants of the global medium were preserved at −20°C. The blastocysts and the embryo culture supernatant were cryopreserved until the transfer cycle. In order to evaluate the configuration of the blastocysts, Gardner's blastocyst grading method was used.[12] The blastocyst grading was assessed by one of 17 experienced embryologists, all trained for 2.5 years. The blastocyst grading assessment was completed 1-2 hours before the embryo transfers, which were performed ~2-3 hours after thawing.

2.2 | Hormone replacement and embryo transfer

In the study cycle, transdermal estradiol (Estraderm M; Kissei, Tokyo, Japan) was used, in combination with a vaginal progesterone suppository (Ultrogestan; Central Apotheke Parfumerie, Basel, Switzerland) for hormone replacement (HR). The preparation of the endometrium was started on day 2 of the HR cycle and was achieved in a step-up regime (2.16-4.32 mg). The progesterone suppository (600 mg/d) was started on day 15. One frozen-thawed blastocyst underwent assisted hatching (AH) and was transferred on day 20 of the HR cycle. Assisted hatching was performed on most blastocysts (>90%), depending on the patient's preference, after careful patient–embryologist consultation. The authors performed the AH for all the patients. In most cases, the AH involved one-quarter of the zona pellucida, using laser shots (ZILOS-tk Laser; Hamilton Thorne, Inc., Beverly, MA, USA), and in a small percentage of cases, the AH involved one-half of the zona pellucida. In all cases, the laser was used for the AH. The embryo transfer was performed transcervically by using an IVF catheter (Fuji Systems, Tokyo, Japan). The embryo culture supernatant, in which the patient's own embryos had been developed, was thawed and injected into the uterine cavity before the frozen-thawed blastocyst transfer on day 17 of the HR cycle. The catheter, loaded with 20 μL of the embryo culture supernatant, was inserted into the uterine cavity and the supernatant was released when the tip of the catheter was ~1 cm from the fundus of the uterine cavity.

2.3 | Time-lapse recordings

The time-lapse recordings were performed by using the EmbryoScope® Time-lapse system (Vitrolife) from June 2013 to December 2014. The

time-lapse images were captured automatically every 15 minutes, at seven focal planes. During the course of this study, the recordings were restricted to patients who were aged between 30 and 40 years, when each yielded more than four oocytes, in order to enable the evaluation of the possible effects of the time-lapse system.

The areas of the male and female pronuclei were analyzed retrospectively by measuring the vertical and horizontal diameters of the pronuclei with the equation: circle area=π×½ vertical diameter×½ horizontal diameter, at various focal plane levels. The measurements were made at 8 hours, 4 hours, and immediately before the PNMBD. The difference in square microns between the two pronuclei in the embryos that resulted in a clinical pregnancy and live birth were compared to the pronuclei of the embryos from the failed pregnancies. The sizes of the male and female pronuclei also were compared in the zygotes in which the female pronuclei were successfully tracked from the polar body (PB) extrusion to the PNMBD by viewing the time-lapse recordings.

2.4 | Statistical analysis

A chi-square test and a student's *t* test were used for the comparison of the proportions. *P*<.05 was considered to be statistically significant.

3 | RESULTS

The average differences in the area between the two pronuclei at 4 hours before and immediately before the PNMBD in the patients who gave birth were 39.9 μm^2 (±37.8) and 11.6 μm^2 (±15.5), respectively. In comparison, the average differences in those that resulted in a failed birth were 62.8 μm^2 (±43.0) and 62.8 μm^2 (±53.3), respectively. Statistically significant differences were obtained when comparing the pronuclear sizes between the patients with successful and unsuccessful births, both 4 hours before (*P*=.012) and immediately before (*P*<.001) the PNMBD. In addition, the average difference between the pronuclear sizes in the patients with a successful birth was significantly smaller when the measurements were made immediately before the PNMBD than 4 hours before the PNMBD (*P*<.001). Such differences were not detected in the group of patients with a failed birth (Figure 1). Measurements could not be obtained in two out of 71 cases due to a lack of clarity of the pictures that had been taken by the time-lapse system and these were eliminated from this study.

Seventy-one frozen-thawed blastocysts were transferred after observations with a time-lapse system at regular intervals, commencing from the zygote stage. In the women who were tracked, the human chorionic gonadotropin (hCG), gestational sac (GS), and fetal heartbeat (FHB) positive (+) rates were 74.2% (52/71), 63.4% (45/71), and 57.7% (41/71), respectively. In the 41 cycles with a FHB (+), 36 women delivered without any chromosomal abnormality in the infant, four women miscarried, and the clinic lost contact with one woman.

Among the 34 live births with successful size measurements in each pronucleus, the largest difference in the area between a pair of pronuclei was 43.9 μm^2. Consequently, the pregnancy rates and live birth rates when the area difference in square microns for a pair of pronuclei was <44 μm^2 and ≥44 μm^2 were compared at 4 hours before and immediately before the PNMBD. When the size difference was assessed immediately prior to the PNMBD and the difference was <44 μm^2, then the hCG (+), GS (+), FHB (+), and live birth rates were 91.7%, 81.3%, 79.1%, and 68.8%, respectively. When
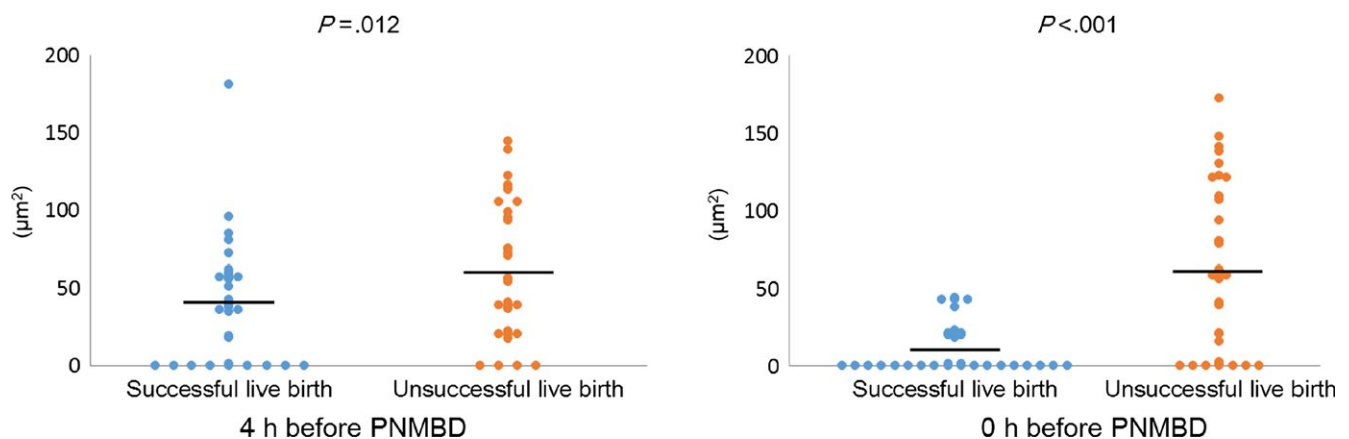
birth	No.	4 h before PNMBD	*P* birth	0 h before PNMBD	*P* birth
Successful live birth	34	39.9 μm^2 (±37.8)	a	11.6 μm^2 (±15.5)	c
Unsuccessful live birth	34	62.8 μm^2 (±43.0)	b	62.8 μm^2 (±53.3)	d

a,c; c,d: *P*<.001 and; a,b: *P*<.01.

FIGURE 1 Average differences in the area between the two pronuclei at 4 hours before and immediately before pronuclear membrane breakdown (PNMBD) in the patients giving birth successfully and in those that resulted in a failed birth

TABLE 1 Various pregnancy parameters and live birth rates in relation to male and female pronuclear area differences

Differences between the two pronuclear areas	Differences in the pronuclear areas 4 hours before PNMBD				Differences in the pronuclear areas 0 hour before PNMBD			
	$<44\ \mu m^2$	*P*	$\geq 44\ \mu m^2$	*P*	$<44\ \mu m^2$	*P*	$\geq 44\ \mu m^2$	*P*
hCG (+) rate (%)	88.9 (32/36)	c	57.6 (19/32)	d	91.7 (44/48)	a	41.7 (5/20)	b
GS (+) rate (%)	75.0 (27/36)		53.1 (17/32)	f	81.3 (39/48)	a	15.0 (3/20)	b,e
FHB (+) rate (%)	69.4 (25/36)		43.8 (14/32)	h	79.1 (38/48)	a	.0 (0/20)	b,g
Live birth rate (%)	61.1 (22/36)		40.6 (13/32)	h	68.8 (33/48)	a	.0 (0/20)	b,g

a,b: *P*<.001; c,d and e,f: *P*<.05; and g,h: *P*<.01. FHB, fetal heartbeat; GS, gestational sac; hCG, human chorionic gonadotropin; PNMBD, pronuclear membrane breakdown.

the difference between the pronuclei was $\geq 44\ \mu m^2$, the hCG (+), GS (+), FHB (+), and live birth rates were 41.7%, 15.0%, .0%, and .0%, respectively. A significant difference was detected in all of the above-mentioned stages of pregnancy and live birth rate (*P*<.001). However, when the difference in the pronuclear size was assessed 4 hours prior to the PNMBD and when the difference was $<44\ \mu m^2$, the results were 88.9%, 75.0%, 69.4%, and 61.1%, respectively. When the difference in the area between the pronuclei was $\geq 44\ \mu m^2$, the results were 57.6%, 53.1%, 43.8%, and 40.6%, respectively. A significant difference was detected only in the HCG (+) comparison (*P*=.012) (Table 1).

Among the 34 live births with successful pronuclear size measurements, 13 female pronuclei could be distinguished from the male pronuclei. The average sizes of the female and male pronuclei 8 hours prior to the PNMBD were 356.3 μm^2 (±48.3) and 404.8 μm^2 (±61.2), respectively. They were 439.4 μm^2 (±60.8) and 463.0 μm^2 (±68.2) 4 hours prior to the PNMBD, respectively, and 494.9 μm^2 (±69.1) and 502.4 μm^2 (±67.1), respectively, immediately prior to the PNMBD. The male pronuclei were significantly larger than the female pronuclei at 8 hours (*P*<.001) and 4 hours (*P*=.049) before the PNMBD, but the differences in their sizes were not significant immediately before the PNMBD (*P*=.15). The difference in size between the female and the male pronuclei diminished as the time to the PNMBD came closer. (Figure 2A). An example of the changes in the size of the female and male pronuclei is shown in Figure 2B.
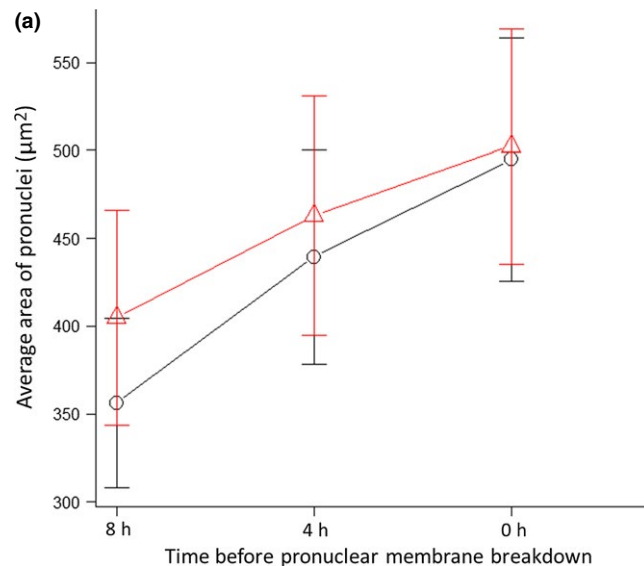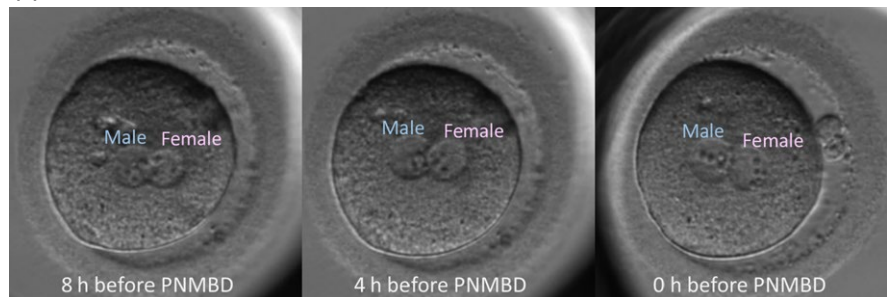

FIGURE 2 A, Average size of the female and male pronuclei, as observed by time-lapse microscopy at 8 hours, 4 hours, and immediately prior to pronuclear membrane breakdown (PNMBD) in the zygotes of women who had a live birth. (○), Female; (△), male. n=13. B, An example of the changes in the size of the female and male pronulei in a zygote that resulted in a live birth

4 | DISCUSSION

In this study, it has been shown that the size of the male and the female pronuclei in the embryos that result in the birth of healthy babies was similar when observed immediately prior to the PNMBD. The presence of two pronuclear areas, confirming normal fertilization, usually is observed 16-18 hours after insemination or ICSI and this corresponds to 4 hours before the PNMBD. However, it was found that the average difference in the pronuclear sizes among the patients with a successful birth was significantly smaller when the measurement was made immediately before the PNMBD, rather than 4 hours before the PNMBD. Thus, measuring the pronuclear sizes by using a time-lapse system immediately prior to the PNMBD provided a more accurate prediction of which embryos would result in the birth of healthy babies.

It is known that a female pronucleus is formed about 1-1.5 hours later than the male pronucleus in the mouse,[13] whereas the delay of the female pronuclear formation is ~20 minutes in the human.[14] The difference could be related to different mechanisms between mouse and human meiosis. For instance, there is an aggregated chromosome phase in the human that lasts for ~1-2 hours after the second PB is extruded. A similar aggregation stage occurs in the human after the first PB extrusion, whereas such changes do not occur in mice.[15] Therefore, animal studies on pronuclear formation do not always apply to humans and the size difference in the male and female pronuclei also can be different between mice and humans.

It should be noted that birth could have occurred during natural conception. This sometimes occurs during frozen-thawed embryo transfer cycles with hormone therapy and such conceptions cannot be eliminated in this study, although the incidence is very small.[10,16] Also, the analysis of the pronuclear size is restricted to 2-D measurements in this study. It is possible that more precise data could be obtained when 3-D analysis becomes possible.

For the last several years, time-lapse observation systems have been useful tools in excluding abnormally cleaved embryos. These systems also have been used in the selection of embryos with a higher potential for the birth of healthy babies. Although at present, precise chromosomal abnormalities, such as mosaic or subchromosomal imbalances, cannot be detected without performing pre-implantation genetic screening or next-generation sequencing, a more accurate evaluation might be possible by accumulating the data and analyzing the embryos from multiple viewpoints.

In this study, the authors focused on successful live birth rates, rather than blastocyst development and pregnancy rates, as >50% of blastocysts are known to have abnormal chromosomal configurations and most of those embryos result in a miscarriage.[17,18] As the difference in size between the female and male pronuclei immediately before the PNMBD is very small among patients with a successful birth, the large difference in size between the female and male pronuclei could be related to chromosomal abnormalities. Moreover, the size of a pronucleus might be correlative with the volume of chromosomes, regardless of the means by which the embryos are derived (ICSI or IVF). For instance, when an abnormally large amount of chromosomes are extruded into the second PB due to chromosome dissegregation, the size of the female pronucleus might become smaller as a result of the small volume of chromosomes in the fertilized oocyte. Although further research is required in order to elucidate this hypothesis, the difference in size between the female and male pronuclei immediately preceding the PNMBD might be a useful measurement in the selection or exclusion of chromosomally normal or abnormal embryos.

DISCLOSURES

Conflict of interest: The authors declare no conflict of interest. *Human and Animal Rights*: This article does not contain any experimental study with human and animal participants that was performed by any of the authors. Informed consent was obtained from all the patients for the usage of their data for publication and this study received the approval of the institutional review board.

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