



# Clinical outcomes of transfer of frozen and thawed single blastocysts derived from nonpronuclear and monopronuclear zygotes

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## Abstract

**Purpose:** In assisted reproductive technology, normal zygotes are bipronuclear (2PN) during fertilization confirmation; however, sometimes, nonpronuclear zygotes (OPN) and monopronuclear zygotes (1PN) are found during routine observations.

**Methods:** To elucidate the clinical usefulness of *in vitro*-fertilized embryos, we investigated the rates of clinical pregnancy, live birth, miscarriage, and congenital abnormality after transfer of frozen-thawed 1PN- and OPN-derived single blastocysts at Denentoshi Ladies Clinic, Kanagawa, Japan.

**Results:** The rates of pregnancy and live birth for 1PN-derived blastocysts obtained by conventional *in vitro* fertilization were 37.5% and 27.1%, respectively, which was not significantly different from those for 2PN-derived blastocysts; however, the rates for OPN-derived blastocysts were significantly lower. The pregnancy and live birth rates for OPN-derived embryos obtained by intracytoplasmic sperm injection (ICSI) were 45.7% and 34.8%, respectively, which was not significantly different from those for 2PN-derived blastocysts; however, the rates for 1PN-derived blastocysts were significantly lower (4.0% for both) than those for 2PN- and OPN-derived blastocysts. No congenital abnormalities were found in infants resulting from transfer of OPN- or 1PN-derived blastocysts.

**Conclusions:** Both 1PN- and OPN-derived blastocysts can be used for embryo transfer; however, care should be taken in making decisions about 1PN-derived blastocysts, especially if they are obtained by ICSI.

## KEYWORDS

abnormal fertilization, clinical outcomes, frozen-thawed blastocyst transfer, monopronuclear zygotes, nonpronuclear zygotes

## 1 | INTRODUCTION

In extracorporeal fertilization methods, fertilization is confirmed by regularly observing the presence of zygotes with two pronuclei (2PN), 16-18 hours after insemination. However, sometimes, zygotes with no pronuclei (OPN) or one pronucleus (1PN) are found, with reported frequencies of 11.3%-20%<sup>1</sup> and 1.6%-7.7%<sup>2</sup>, respectively. Such embryos are considered to be unfertilized or abnormally fertilized ova, and therefore, they are not suitable for clinical use and are discarded. However, such embryos sometimes undergo cleavage similar to normally fertilized 2PN-derived embryos<sup>3</sup>. Chromosomal analysis has shown that some 1PN- and OPN-derived embryos are diploid and have normal chromosomal structure<sup>4,5</sup>. Furthermore, births of healthy infants resulting from the transfer of such blastocysts have been reported<sup>6,7</sup>.

To investigate the clinical usefulness of 1PN- and OPN-derived embryos, a retrospective study was carried out. Clinical pregnancy, live birth, miscarriage, and congenital abnormality rates after transfer of frozen-thawed 1PN- and OPN-derived single blastocysts were studied.

## 2 | MATERIALS AND METHODS

### 2.1 | Patients

This study covered 11588 female reproductive cycles, including both natural and hormone-replacement cycles, during which frozen-thawed single-blastocyst transfer was carried out at Denentoshi Ladies Clinic, Kanagawa, Japan, between January 2006 and December 2015. All women involved were aged 41 or younger at the time of oocyte retrieval. All embryos included in the study were observed periodically, and the clinical pregnancy, live birth, miscarriage, and congenital abnormality rates were compared retrospectively between fertilization methods and among 2PN-, 1PN-, and OPN-derived blastocysts. *In vitro* fertilization (IVF) was carried out with 2PN-, 1PN-, and OPN-derived blastocysts in 4942, 48, and 38 cycles, whereas intracytoplasmic sperm injection (ICSI) was carried out in 6489, 25, and 46 cycles, respectively.

### 2.2 | Ovarian stimulation

Ovulation was induced by mild stimulation from day 3 of menstruation by oral administration of clomifene (Clomid<sup>®</sup>; Fuji Pharmaceutical, Tokyo, Japan) and administration of follicle-stimulating hormone (Follistim<sup>®</sup>; MSD, Tokyo, Japan or Gonal F<sup>®</sup>; Merck, Darmstadt, Germany) or human menopausal gonadotropin (HMG Teizo<sup>®</sup>; Aska Pharmaceutical, Tokyo, Japan, HMG Fuji<sup>®</sup>; Fuji Pharmaceutical, Tokyo, Japan, HMG Kowa<sup>®</sup>; Kowa Company, Aichi, Japan, or HMG Ferring<sup>®</sup>; Ferring Pharmaceuticals, Tokyo, Japan). If necessary, a gonadotropin-releasing hormone antagonist (Cetrotide<sup>®</sup>; Merck Serono, Tokyo, Japan or Ganirest<sup>®</sup>; MSD, Tokyo, Japan) was also administered. When the mean diameter of the dominant follicle on two axes reached at least 18 mm, a human chorionic gonadotropin (hCG

Fuji<sup>®</sup>; Fuji Pharmaceutical, Tokyo, Japan) or gonadotropin-releasing hormone agonist (Busrecur<sup>®</sup>; Fuji Pharmaceutical, Tokyo, Japan) was administered; oocyte pick-up was performed 34-36 hours later.

### 2.3 | Embryo culture

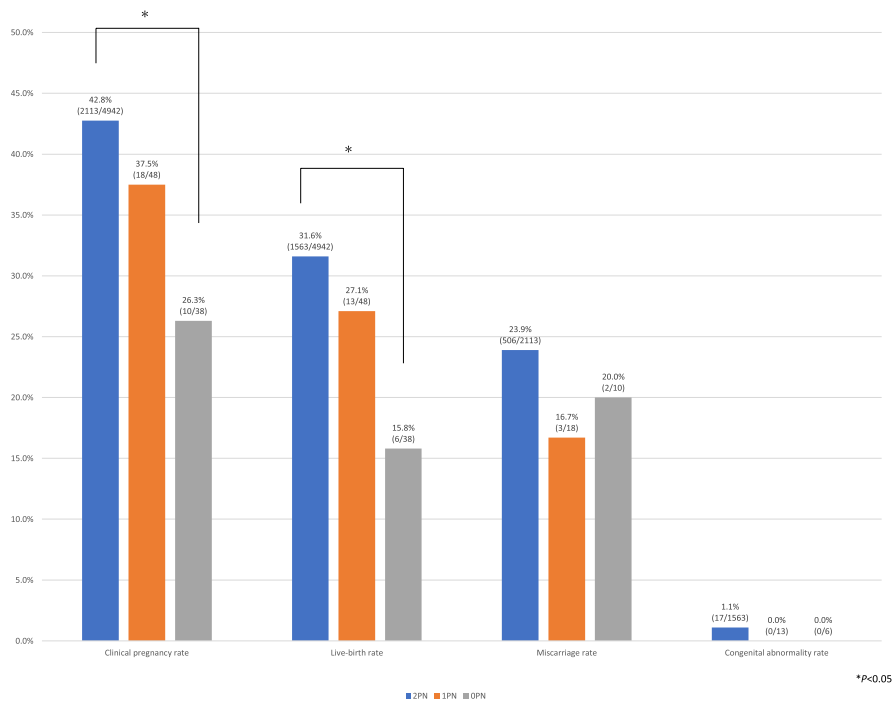
After IVF or ICSI, embryo culture was carried out in an atmosphere of 6% carbon dioxide, 5% oxygen, and 89% nitrogen. Until confirmation of fertilization, embryos were cultured in Universal IVF Medium (Cooper Surgical companies, Trumbull, USA), whereas after confirmation, Global Medium (Cooper Surgical companies, Trumbull, USA) or Sydney IVF fertilization medium (Cook Medical, Bloomington, USA) and Blast Assist System media 1 and 2 (Cooper Surgical companies, Trumbull, CT, USA) were used. Fertilization was confirmed 16-18 hours after insemination by examining the ova using a stereomicroscope and by checking the pronuclei and polar bodies. 2PN was defined as the presence of two clearly distinct pronuclei and two polar bodies. 1PN was defined as the presence of only one pronucleus and two polar bodies. OPN was defined as the absence of pronuclei and presence of two polar bodies.

### 2.4 | Cryopreservation

The embryos in this study had Gardner grades of 3BB or higher when cryopreserved on day 5 or 6. The criteria for cryopreservation of 1PN and OPN embryos were the same as those for 2PN embryos. Cryopreservation was carried out by vitrification using vitrification medium (Kitazato Corporation, Shizuoka, Japan), and embryos were thawed in thawing medium (Kitazato Corporation, Shizuoka, Japan). After thawing, assisted hatching was carried out on all embryos by partial zona dissection.

### 2.5 | Frozen-thawed blastocyst transfer cycle

Before transfer of thawed embryos during the natural cycle, either natural or induced ovulation was confirmed, and five days later, frozen-thawed single-blastocyst transfer was performed. For transfer of thawed embryos during the hormone-replacement cycle, estradiol (Estrana<sup>®</sup>; Hisamitsu Pharmaceutical, Tokyo, Japan) was administered from approximately day 3 of menstruation, and after the endometrial thickness was confirmed to be at least 8 mm, progesterone (Luteum Vaginal Suppository<sup>®</sup>; Aska Pharmaceutical, Tokyo, Japan or Lutinus<sup>®</sup>; Ferring Pharmaceuticals, Tokyo, Japan) was administered. Frozen-thawed single-blastocyst transfer was carried out on day 6 after initiation of progesterone administration using an IVF Catheter (Fuji Systems Corporation, Tokyo, Japan) or an ET Catheter (Kitazato Corporation, Shizuoka, Japan) with ultrasound guidance. When transplanting embryos, 2PN embryos were given priority, regardless of the Gardner grade. 1PN and OPN embryos were transplanted after informed consent into patients without 2PN who were not planning to undergo oocyte pick-up thereafter. Patients were considered clinically pregnant upon confirmation of the presence of a gestation sac by transvaginal ultrasonography. Congenital abnormalities were defined as developmental abnormalities originating during the embryonic and fetal periods.



**FIGURE 1** Comparison of the outcomes for 2PN-, 1PN-, and OPN-derived blastocysts obtained by conventional in vitro fertilization (IVF)

Informed consent from the patients was obtained in accordance with the Denentoshi Ladies Clinic's code of ethics before their participation in the study.

## 2.6 | Statistical analysis

For statistical evaluation of mean ages, a multiple comparison test was carried out using the Steel-Dwass method, with a statistical significance level of  $P < 0.05$ . For statistical evaluation of the clinical pregnancy, live birth, miscarriage, and congenital abnormality rates, the chi-square test was used, with a statistical significance level of  $P < 0.05$ .

## 3 | RESULTS

No significant inter-group differences in the mean ages of the patients were found. Comparison of the outcomes for 2PN-, 1PN-, and OPN-derived blastocysts obtained by IVF showed that the clinical pregnancy rates were 42.8% (2113 out of 4942), 37.5% (18 out of 48), and 26.3% (10 out of 38); the live birth rates were 31.6% (1563 out of 4942), 27.1% (13 out of 48), and 15.8% (6 out of 38); the miscarriage rates were 23.9% (506 out of 2113), 16.7% (3 out of 18), and 20.0% (2 out of 10); and the congenital abnormality rates were 1.1% (17 out of 1563), 0.0% (0 out of 13), and 0.0% (0 out of 6), respectively. The clinical pregnancy and live birth rates were significantly different between 2PN- and OPN-derived blastocysts ( $P < 0.05$ ) (Figure 1). The miscarriage rates and the congenital abnormality rates were not different between three groups. The result of abnormalities at birth in infants from 2PN embryos was two cases of Hydronephrosis, 1 case of Hydronephrosis with Pneumothorax, Pneumomediastinum, Congenital heart disease with Asplenia syndrome, Ventricular septal defect, Atrioventricular

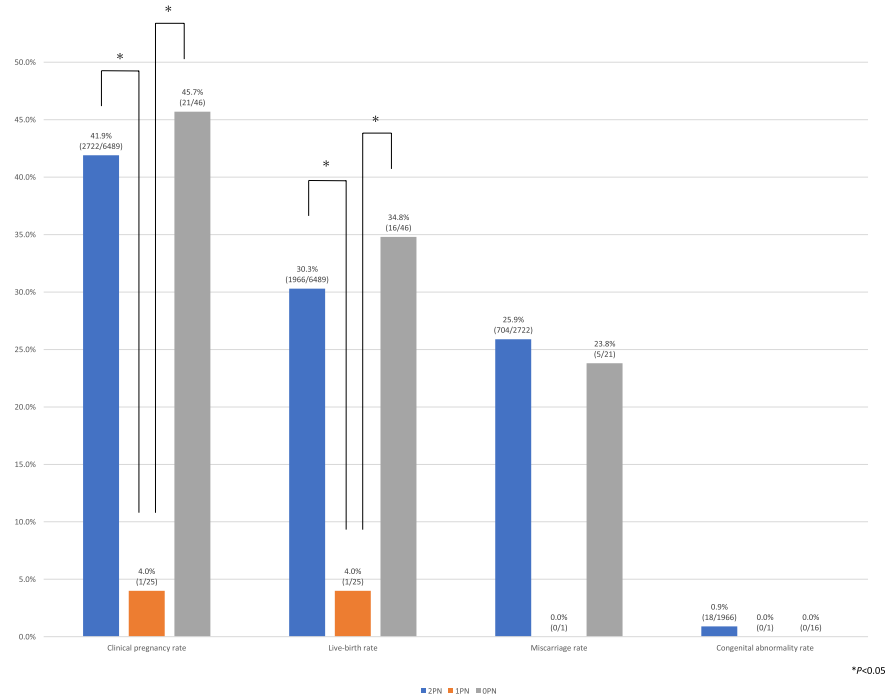
septal defect, Ankyloproctia with Thumb polydactyly, Symphalangism, Polydactyly, Hypoplastic left heart syndrome, Microtia with Hypoplastic lower category and Dysacusia, Inguinal hernia, Congenital diaphragmatic hernia, Strawberry mark, Accessory ear, Aortic coarctation, and 18Trisomy. No abnormalities were observed at birth in infants resulting from 1PN and OPN embryos.

Comparison of the outcomes for 2PN-, 1PN-, and OPN-derived blastocysts obtained by ICSI showed that the clinical pregnancy rates were 41.9% (2722 out of 6489), 4.0% (1 out of 25), and 45.7% (21 out of 46); the live birth rates were 30.3% (1966 out of 6489), 4.0% (1 out of 25), and 34.8% (16 out of 46); the miscarriage rates were 25.9% (704 out of 2722), 0.0% (0 out of 1), and 23.8% (5 out of 21); and the congenital abnormality rates were 0.9% (18 out of 1966), 0.0% (0 out of 1), and 0.0% (0 out of 16), respectively. The clinical pregnancy and live birth rates were significantly different between 2PN- and 1PN-, and between 1PN- and OPN-derived blastocysts ( $P < 0.05$ ) (Figure 2). The miscarriage rates and the congenital abnormality rates were not different between three groups. The result of abnormalities at birth in infants from 2PN embryos was four cases of Ventricular septal defect, two cases of Hydronephrosis, 1 case of Mild cretinism, Left ear microotitis, Biliary dilatation, Ankyloproctia, Complete transposition of great vessel, Head sebaceous nevus, Hypoplasia of lung, Lymphangioma, Atresia oesophagi with Symphalangism and Cleft palate, Cleft lip with cleft palate, Symphalangism, and Arteriovenous malformation. No abnormalities were observed at birth in infants resulting from 1PN and OPN embryos.

## 4 | DISCUSSION

In the present study, the clinical outcomes of transfers of frozen-thawed single blastocysts obtained from 1PN- and OPN-derived

**FIGURE 2** Comparison of the outcomes for 2PN-, 1PN-, and OPN-derived blastocysts obtained by intracytoplasmic sperm injection (ICSI)



blastocysts were compared to those achieved with 2PN-derived blastocysts. We found that the outcomes for 1PN-derived blastocysts obtained by IVF and OPN-derived blastocysts obtained by ICSI were similar to those achieved with 2PN-derived blastocysts.

1PN may result from (a) inappropriate timing of pronuclear formation and/or disappearance of pronuclei<sup>8-10</sup>, (b) fusion of paternal and maternal pronuclei<sup>11,12</sup>, or (c) parthenogenetic development of either paternal or maternal material<sup>13,14</sup>. Parthenogenetic development results in haploid cells, whereas (a) and (b) result in a normal chromosome number. Embryos with a normal chromosome number are present even among 1PN. In a previous study, 1PN-derived embryos obtained by IVF and ICSI contained 86.7% and 30.3% of both the maternal and paternal genomes, respectively<sup>14</sup>. In addition, the diploid rate of 1PN-derived embryo chromosomes reportedly is significantly higher in IVF than in ICSI embryos<sup>4,13-15</sup>. Otsu et al, reported that all 1PN-derived blastocysts obtained by IVF were diploid<sup>16</sup>. Furthermore, the haploid rate of 1PN-derived embryos is higher when they are obtained by ICSI than when they are obtained by IVF<sup>13-15</sup>. Sultan et al, reported that Y-chromosome prevalence in 1PN was low (10%), and in most cases, the development was parthenogenetic<sup>4</sup>. Furthermore, chromosomal aberrations were found in all analyzed cases of 1PN obtained by ICSI<sup>17</sup>. Levron et al, suggested that 1PN embryos obtained by IVF are possibly formed through enclosure of the paternal and maternal chromosomes by a single nuclear membrane<sup>11</sup>. During IVF, sperm reportedly enter the oolemma from various directions, including positions close to the spindle apparatus in the ovular cytoplasm<sup>18</sup>. Thus, the probability of the paternal and maternal chromosomes being in close proximity before nuclear membrane formation is higher with IVF than with ICSI, suggesting that diploid 1PN will be obtained more frequently by IVF than by ICSI. In a study of ICSI in mice, Krukowska et al,

showed that injection of sperm into the ovular cytoplasm close to the spindle apparatus resulted in the fusion of paternal and maternal chromosomes before nuclear membrane formation; the paternal and maternal chromosomes were thus enclosed in a single nuclear membrane<sup>19</sup>. Therefore, diploid 1PN-derived embryos obtained by ICSI may develop by the same mechanism as those obtained by IVF. It is expected that the spindle apparatus can be avoided when sperms are injected; therefore, the frequency of this phenomenon in ICSI is probably lower than that in IVF. As a result, the clinical outcomes for 1PN embryos obtained by ICSI are very poor; however, when 1PN embryos are obtained by IVF, the clinical outcomes are nearly the same as those for 2PN embryos.

Births of healthy infants after transfer of 1PN-derived embryos have been reported<sup>1,6,20,21</sup>, but most of these were embryos obtained by IVF. The present study included only one case of a live birth from a 1PN blastocyst obtained by ICSI. Although the infant did not have congenital abnormalities, one case does not provide sufficient evidence for the safety of 1PN-derived blastocyst transfer, and thus, further research is required.

OPN may result from (a) the lack of formation of a pronucleus, (b) disappearance of the pronucleus, or (c) an unfertilized ovum. In the case of lack of formation of a pronucleus or disappearance of the pronucleus, the fertilization process might be normal, but the cell cycle might be faster or slower than usual, making confirmation of the presence of a pronucleus impossible. In a previous study, time-lapse observation after ICSI showed that the pronucleus can appear after up to 30.14 hours after sperm injection and can disappear as early as 6.16 hours after injection<sup>22</sup>. This suggests that in cases where fertilization was confirmed within 16-18 hours, the lack of pronuclei might be due to disappearance of pronuclei before confirmation of fertilization and/or the development of pronuclei after confirmation.

In the present study, when OPN was obtained by ICSI, the clinical outcome was nearly the same as that for 2PN, but when OPN was obtained by IVF, the clinical outcome was worse than that for 2PN. Li et al, previously compared the implantation rates of frozen-thawed OPN-derived blastocysts obtained by different fertilization methods; they found a difference, albeit not significant, between IVF and ICSI, at 35.59% (21 out of 59) and 45.45% (15 out of 33), respectively<sup>7</sup>. The results of the present study were similar. Fancsovsits et al, reported that early pronuclear disappearance is a good indicator for the selection of appropriate embryos and that the rate was higher, albeit not significantly, when obtained by ICSI than when obtained by IVF, at 41.1% and 37.1%, respectively<sup>23</sup>. The timing of fertilization in IVF was unclear, whereas this timing was clear in ICSI. For ICSI, mature ova are used, whereas in IVF, ova have varying degrees of maturity. In general, in the mature ovum, the pronucleus appears 16-18 hours after insemination. Thus, if ovular maturation is delayed, fertilization may also be delayed. Therefore, it is probable that the proportion of OPN embryos in which the pronucleus disappears before confirmation of fertilization is greater in embryos obtained by ICSI than in those obtained by IVF. This difference was reflected in the clinical outcomes in the present study. However, in the majority of normal fertilized embryos, the pronucleus does not disappear 16-18 hours after insemination. Even if such embryos coexist, their proportion is extremely small. Nagy et al, confirmed fertilization over time after insemination, and 99% (92 out of 93) of 2PN embryos had observable pronuclei after 16 hours<sup>9</sup>. In this study, OPN embryos may include embryos in which pronuclei appeared or disappeared before and after fertilization was confirmed; however, the proportion of such embryos is considered infinitely small.

According to chromosome analysis, 44.4%-57.0% of OPN embryos are diploid<sup>5,24</sup>. Noyes et al, reported that the diploidy rate in OPN-derived embryos on day 3 was only 3%, and the blastocyst stage was not reached even when OPN culture was extended<sup>25</sup>. All embryos included in the present study were frozen when they reached the blastocyst stage, and were therefore different from the embryos studied by Noyes et al, Previous reports have indicated that blastocyst culture is effective for selecting embryos with normal chromosomes among 1PN- and OPN-derived embryos<sup>5-7</sup>, and it is probable that in the present study, OPN-derived embryos with aberrant chromosomes were eliminated. Li et al, found that the implantation rate was lower for OPN-derived embryos than for 2PN-derived embryos when frozen-thawed cleavage-phase transfer was performed. However, the implantation rates were nearly the same with blastocyst transfer<sup>7</sup>. Liao et al, reported a significantly higher diploidy rate for 1PN-derived blastocysts (74.6%) than for 1PN with halted cleavage (31.6%)<sup>26</sup>. In previous reports, the fractions of embryos reaching the blastocyst stage were low, 16.7%-35.0%, for OPN<sup>5,7</sup> and 3.6%-21.4% for 1PN<sup>5,6,16</sup>, suggesting that embryos with aberrant chromosomes may be eliminated during the blastocyst culture process. However, Liao et al, reported that elimination of haploids, but not mosaic embryos and polyploids, is possible by blastocyst transfer<sup>26</sup>, showing that the

safety of 1PN or OPN embryos cannot be ensured simply based on blastocyst stage.

Fertilization, pronuclei, and polar bodies were checked using a stereomicroscope. Fertilization assessment, among other embryology assessments, requires strong skills and expertise. Fertilization was confirmed after rolling the embryo using a Pasteur pipette. However, artificial pronuclei and polar bodies might have been overlooked in this assessment.

Although the clinical outcome differed between 1PN and OPN depending on the fertilization method, live births were achieved with both methods, and no congenital abnormalities were found. In addition, the clinical outcomes for 1PN embryos obtained by IVF and OPN embryos obtained by ICSI were nearly the same as those for 2PN embryos. This finding might broaden the range of opportunities for pregnancy, as it suggests it is possible to transfer OPN- or 1PN-derived embryos into patients from whom 2PN-derived embryos could not be obtained and for whom obtaining 2PN embryos in future would be difficult. However, as reported previously, transfer of 1PN- and OPN-derived embryos is uncommon; therefore, the numbers of subjects undergoing transplant of such embryos in this study were limited, and we could not predict the safety of using these embryos. Thus, any patient opting for this approach will have to give satisfactory informed consent. In addition, further detailed studies are required to investigate whether it is safe to transfer 1PN-derived embryos obtained by ICSI. Although no congenital anomalies were observed in this study, the safety of this approach is unknown given the few cases of 1PN transplanted embryos.

## HUMAN/ANIMAL RIGHTS STATEMENT AND INFORMED CONSENT

This study was approved by an institutional ethics committee. This article does not contain any studies with human or animal subjects performed by any of the authors. Informed consent was obtained from all the patients in this study.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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