J Assist Reprod Genet (2015) 32:1401–1407
DOI 10.1007/s10815-015-0518-y

CASE REPORT

Birth of nine normal healthy babies following transfer of blastocysts derived from human single-pronucleate zygotes

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Received: 2 April 2015 / Accepted: 15 June 2015 / Published online: 26 June 2015

Abstract
Purpose The purpose of this study is to examine the clinical outcomes of blastocysts derived from human single-pronucleate (1PN) embryos after conventional in vitro fertilization (cIVF) and intracytoplasmic sperm injection (ICSI) cycles.
Methods This was a retrospective study at a reproductive center of a hospital. To evaluate embryo quality and clinical outcomes, cIVF or ICSI cycles with one or more 1PN embryos were compared with same cycles with 2PN embryos (control cycles).
Results A total of 623 cycles (426 cIVF cycles and 197 ICSI cycles) were treated with cIVF or ICSI. The single pronuclear status rate was similar between cIVF (22.1 %) and ICSI (25.1 %) cycles. Although the development rates of 1PN embryos on day 3 and day 5/6 in cIVF were significantly higher than those in ICSI, those of 1PN embryos in cIVF were significantly lower compared to 2PN embryos ($p<0.01$). Nonetheless, the ongoing pregnancy rates achieved with 1PN blastocysts in 1PN embryos did not significantly differ from the control group. Thirty-three transfer cycles with 33 blastocysts derived from 1PN embryos in cIVF resulted in nine deliveries with no newborn malformations; however, no implantation was observed in three ICSI cycles.
Conclusion Although the blastocyst formation rate of 1PN embryos was significantly lower than 2PN embryos in cIVF and ICSI cycles, 1PN blastocysts in cIVF, and not from ICSI, demonstrated an adequate ongoing pregnancy rate. These results suggested that 1PN blastocysts in cIVF are available for clinical use and may lead to an increase in the chance of pregnancy in patients receiving assisted reproductive technology with 1PN embryos.

Capsule It may be possible to expect an adequate ongoing pregnancy rate after blastocyst transfer derived from 1PN zygotes in cIVF cycles but not in ICSI cycles.

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Keywords Single-pronucleate embryos · Conventional in vitro fertilization · Intracytoplasmic sperm injection · Blastocyst culture · Clinical outcome · Normal healthy babies

Abbreviations

ART	Assisted reproductive technology
IVF	In vitro fertilization
ICSI	Intracytoplasmic sperm injection
F-TBT	Frozen-thawed blastocyst transfer

Introduction

In human-assisted reproductive technology (ART), approximately 1.6–7.7 % of embryos show abnormal zygotic stages with a single pronucleus (1PN) following conventional in vitro fertilization (cIVF) or intracytoplasmic sperm injection (ICSI) [1–6]. Although such abnormal pronuclear-stage embryos are considered unsuitable for transfer to patients, they are occasionally transferred and a few attempts for normal pregnancies have been reported including embryos derived from 1PN zygotes [4, 5, 7, 8]. However, formation rate and clinical pregnancy rate of blastocysts derived from 1PN zygotes were unclear. Recently, the analyses of chromosomal status and genetic composition in 1PN zygotes and embryos have been reported. For example, when the chromosomal status of 1PN embryos were analyzed using fluorescence in situ hybridization (FISH), the rate of diploid chromosome constitution of the cleavage-stage embryos and blastocysts derived from 1PN in cIVF were significantly higher than in ICSI [6, 9, 10]. Furthermore, van der Heijden et al. reported that the genetic composition of 1PN zygotes was observed based on histon modification using immunostaining, and some of these zygotes included the maternal and paternal genome in 1PN [11]. Further, they reported that the rate of diploid chromosomes in 1PN zygotes were 86.7 % in cIVF and 30.3 % in ICSI [11]. Moreover, Liao et al. reported that the diploid rate of blastocysts derived from 1PN embryos was significantly higher than in arrested cleavage-stage embryos [12]. Although there have been several reports analyzing the chromosome constitution of embryos derived from 1PN by FISH or immunostaining, there is little embryological and clinical data for 1PN embryos. The strongest evidence of good-quality embryos is the production of live healthy babies. In this study, we retrospectively studied the clinical outcomes of 1PN embryos which were cultured until blastocyst stage in cIVF and ICSI cycles. To evaluate the 1PN embryos, all 1PN embryos were analyzed for the blastocyst formation rate and good-quality blastocyst rate; some 1PN blastocysts were transferred into patients to examine their potential for development into healthy babies. The cycles in which at least one 1PN embryo was observed (1PN groups) were compared to cycles with 2PN embryos (control groups) obtained in the same cIVF or ICSI cycles.

Materials and methods

Patients

In this study, a total of 623 patients were included who underwent cIVF/ICSI at a private clinic [Department of Infertility, Angel Bell Hospital (Aichi Prefecture, Japan)] between May 2010 and September 2014. All patients underwent cIVF/ICSI, embryo culture until blastocyst stage, and blastocyst transfer onto an endometrium prepared using hormone therapy. Cycles with split (cIVF + ICSI) were excluded. We avoided transferring blastocysts derived from 1PN zygotes as much as possible, since the safety of these embryos has not been established. Consequently, these embryos we only transferred when there were no other embryos available.

Ovarian stimulation

Ovarian stimulation, oocyte retrieval, and cIVF/ICSI procedures have been previously described by Murata et al. [13]. Most often, the ovarian stimulation protocol entailed administration of a gonadotrophin-releasing hormone (GnRH) antagonist cetrolelix (Cetrotide; Merck Serono) with subsequent addition of recombinant follicle stimulating hormone (FSH; Gonal-F®, Merck Serono)/human menopausal gonadotrophin (hMG; Menopur; Ferring Pharmaceuticals). The exogenous FSH/hMG was administered daily until the leading follicles averaged 18 mm in diameter. Measurements were made using serial ultrasound recordings. In addition to exogenous FSH/hMG administration, cetrorelix was injected daily once the leading follicle reached 16 mm in diameter or a premature luteinizing hormone (LH) surge was suspected based on serum LH monitoring. These injections were continued until the day before the human chorionic gonadotrophin (hCG; HCG Mochida, Mochida Pharmaceutical, Tokyo, Japan) was injected.

Alternatively, ovarian stimulation entailed administration of a GnRH agonist (buserelin acetate; Sprecure, Mochida Pharmaceutical) in a short (flare-up) protocol with subsequent addition of FSH/hMG. In another cycle, clomiphene citrate with/without exogenous FSH/hMG was used for follicular growth induction.

hCG (5000 IU) was administered 36 h before transvaginal oocyte retrieval or a GnRH agonist was administered twice, 35 and 36 h, before retrieval. Following retrieval, oocytes were fertilized using cIVF or ICSI.

Fertilization, embryo culture and evaluation, and cryopreservation

Fertilization was performed 3–6 h after retrieval in Universal IVF medium (ORIGIO Japan, Yokohama, Japan) with either cIVF or ICSI as appropriate for the presence or absence of male infertility factor. After cIVF or ICSI, the oocytes were cultured in individual 20-μL droplets of universal IVF medium on petridishes (60 mm; Falcon) at 37 °C under a humid atmosphere of 6 % CO_2, 5 % O_2, and 89 % N_2. Fertilization was assessed 18–20 h after cIVF or ICSI based on the presence of two pronuclei. The zygotes were divided and cultured as 2PN or 1PN on culture dishes (4-well plastic dish; Nunc) containing 100 μL of culture medium (Single-step medium;

global total medium, LifeGlobal), covered with mineral oil (light mineral oil, Irvine Scientific) and cultured until day 5/6.

Evaluation of embryos on day 3 was based on the number and symmetry of the blastomeres and the percentage fragmentation and multinucleation. Blastocysts were evaluated by two embryologists who assigned each blastocyst a grade using the grading system of Schoolcraft et al. [14]. Embryos were cryopreserved on either day 5 or 6, depending on the grade of the blastocyst morphology. Cooling and embryo warming were performed using the Cryotop methodology for human embryo vitrification described by Kuwayama et al. [15]. Equilibration, vitrification, warming, dilution, and washing solutions were provided in a vitrification kit (VT-101 and VT-102; Kitazato Corporation, Shizuoka, Japan).

Frozen-thawed blastocyst transfer cycle

All frozen-thawed blastocyst transfer (F-TBT) cycles were performed during artificial hormone replacement cycles with identical endometrial preparation protocols [13]. The proliferative phase was induced by transdermal administration of incremental doses of estradiol (E2) (Estrana; Hisamitsu Pharmaceutical, Tokyo, Japan). After confirming the endometrial thickness to be more than 8 mm using ultrasonography, daily administration of 6 mg of chlormadinone acetate (Lutoral; Shionogi & Co., Ltd., Tokyo, Japan) was initiated, and blastocyst transfer was performed on the 5th or 6th day of chlormadinone administration. Serum hCG was checked 9 days after the blastocyst transfer, and patients with hCG >50 IU/L were considered pregnant. One week later, transvaginal ultrasound was performed to confirm intrauterine pregnancy. In cases of pregnancy, transdermal E2 (2.16 mg/every 2 days) and chlormadinone (6 mg/day) were administered for the first 9 weeks of gestation. Fetal viability was then assessed after 3 weeks, and ongoing pregnancy was determined after 12 weeks of pregnancy. The implantation rate was determined by dividing the number of gestational sacs by the number of embryos transferred.

Statistical analysis

The distribution of variable data was assessed, and the normal and non-normal distributions were appropriately treated. Data were presented as means±standard deviation. Group characteristics were compared using the Mann–Whitney U test. The χ^2 test was used to evaluate the significance of the proportion (%). Values of $p<0.05$ were considered significant.

Results

Figure 1 summarized the flow chart of this study. A total of 623 cycles were treated with cIVF or ICSI (426 cIVF and 197 ICSI cycles) between May 2010 and September 2014 that included at least one 1PN embryo in our clinic. In all cycles, the incidence of 1PN formation was 5.7 % (576/10,141) in cIVF and 4.7 % (252/5325) in ICSI.

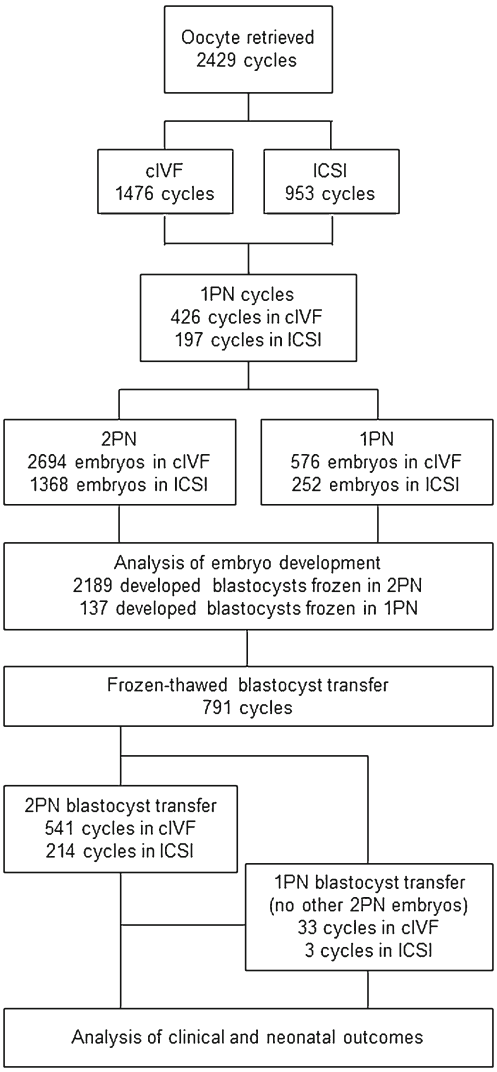

Fig. 1 Flow chart of cIVF/ICSI cycles and embryo development in the study

As shown in Table 1, the patient characteristics and the pronuclear status after cIVF or ICSI differed significantly with respect to the mean number of previous oocyte retrievals, 2PN rate, and the mean number of cryopreserved blastocysts of 1PN. On the other hand, the rate of 1PN was similar between the cIVF (22.1 %) and ICSI (25.1 %) cycles.

As shown in Table 2, after culturing the cIVF- or ICSI-derived 1PN embryos for 3 days, the development rates of the good-quality embryos did not significantly differ (19.8 vs. 17.5 %, respectively). When 1PN embryos were cultured to blastocyst, the blastocyst formation rate and good-quality blastocyst rate in the cIVF cycles (21.4 and 7.7 %) was significantly higher than those in the ICSI cycles (10.7 and

Table 1 Patient characteristics in cIVF and ICSI cycles including at least one 1PN embryo

Cycles	cIVF	ICSI	*p* value
No. of cycles	426	197	–
Age (years)	36.0±4.2	36.0±3.9	NS
Previous oocyte retrieval	1.7±1.3	2.6±2.7	<0.001
Dose of FSH (IU/mL)	895.0±537.1	846.6±575.8	NS
Dose of hMG (IU/mL)	444.2±490.1	444.7±451.6	NS
Serum E2 concentration (pg/mL)	1673.2±1303.6	1651.3±1218.0	NS
Serum P4 concentration (ng/mL)	1.0±0.7	0.9±0.6	NS
Serum LH concentration (IU/L)	5.0±7.0	6.3±11.8	NS
No. of retrieved oocytes	5034	2383	–
Mean no. of retrieved oocytes	11.8±7.9	12.1±9.2	NS
No. of MII oocytes	4019	1864	–
% MII oocytes/retrieved oocytes (%)	81.4±15.9	80.7±16.6	NS
Mean no. of MII oocytes	9.4±6.2	9.5±7.2	NS
No. of 2PN	2694	1368	–
% 2PN/MII (%)	60.2±23.3	62.8±25.8	<0.05
Mean no. of 2PN	6.3±5.0	6.9±6.3	NS
No. of 1PN	576	252	–
% 1PN/MII (%)	22.1±20.5	25.1±25.0	NS
Mean no. of 1PN	1.4±0.9	1.3±0.6	NS
Mean no. of cryopreserved blastocysts of 2PN	3.3±3.2	3.4±3.9	NS
Mean no. of cryopreserved blastocysts of 1PN	0.4±0.7	0.2±0.4	<0.001

Data are shown as the means±standard deviation for each group, and the statistical significance of differences between group characteristics were assessed using the Mann–Whitney *U* test. Values of $p<0.05$ were considered significant

cIVF conventional in-vitro fertilization, *ICSI* intracytoplasmic sperm injection, *dose of FSH* exogenous FSH administration, *dose of hMG* exogenous hMG administration, *serum E2 concentration* serum E2 concentration on the day of hCG administration, *serum P4 concentration* serum P4 concentration on the day of hCG administration, *serum LH concentration* serum LH concentration on the day of hCG administration, *MII* metaphase II, *2PN* two pronuclei, *1PN* one pronucleus

0.7 %) ($p<0.01$). However, blastocyst formation rate derived from 1PN embryos were significantly lower compared to 2PN embryos ($p<0.01$).

After culturing 1PN embryos derived from cIVF and ICSI to blastocysts, these blastocysts were cryopreserved until F-TBT was performed (Table 3). F-TBT was performed in 791

Table 2 Embryological outcomes of 2PN and 1PN zygotes in cIVF and ICSI cycles

Insemination	cIVF		ICSI	
No. of cycles	426		197	
Pronuclear state	2PN	1PN	2PN	1PN
No. of embryos	2694	576	1368	252
% good-quality embryos on day 3	37.3 ± 30.4^{a}	19.8 ± 37.7^{b}	37.8 ± 31.6^{a}	17.5 ± 35.8^{b}
% blastocysts on day 5	55.7 ± 30.8^{ac}	21.4 ± 38.9^{bc}	46.4 ± 31.5^{ad}	10.7 ± 29.3^{bd}
% good-quality blastocysts on day 5	26.6 ± 26.6^{ac}	7.7 ± 25.0^{bc}	19.3 ± 21.7^{ad}	0.7 ± 7.5^{bd}
% good-quality blastocysts on day 5/6	29.7 ± 27.6^{ac}	9.6 ± 27.5^{bc}	22.5 ± 23.2^{ad}	1.4 ± 10.9^{bd}
% cryopreserved blastocysts/embryos	55.4 ± 29.3^{ac}	20.3 ± 38.3^{bc}	47.9 ± 29.6^{ad}	9.8 ± 27.7^{bd}

The embryological outcomes were compared between 1PN and 2PN embryos within each cycle in cIVF or ICSI. Data are shown as the means±standard deviation for each group, and the statistical significance of differences between group characteristics were assessed using the Mann–Whitney *U* test. Values of $p<0.05$ were considered significant. Good-quality embryos on day 3=7–9 cells with ≤10 % fragmentation, good-quality blastocysts= blastocyst expansion grade 3≤, inner cell mass grade B≤, trophectoderm grade B≤. [a] vs. [b], [c] vs. [d]; values with different superscript letters are significantly different ($p<0.05$)

Table 3 Clinical outcomes of 1PN and 2PN blastocysts in cIVF and ICSI cycles

Insemination	cIVF		ICSI	
Pronuclear state	2PN	1PN	2PN	1PN
No. of cycles	541	33	214	3
No. of transferred blastocyst	675	33	273	4
Mean no. of transferred blastocyst	1.2	1.0	1.3	1.3
No. of positive for βhCG (%)	234 (43.3)	14 (42.4)	103 (48.1)	0 (0.0)
No. of implantations (%)	211 (39.0)	11 (33.3)	100 (46.7)	0 (0.0)
No. of clinical pregnancies (%)	194 (35.9)	11 (33.3)	88 (41.1)	0 (0.0)
No. of spontaneous abortions (%)	42 (21.6)	2 (18.2)	17 (19.3)	0 (0.0)
No. of ongoing pregnancies (%)	152 (28.1)	9 (27.3)	71 (33.2)	0 (0.0)

Positivity for βhCG in serum was checked 9 days after blastocyst transfer, and patients with hCG>50 IU/L were considered pregnant. Implantation rate was calculated as the number of gestational sacs/number of transferred embryos∗100. Clinical pregnancy was confirmed as at least one gestational sac seen within the uterus on transvaginal ultrasound. Spontaneous abortions were confirmed as the number of spontaneous abortions/number of clinical pregnancies. Ongoing pregnancy was determined after 12 weeks of pregnancy. The chi-square test was used to evaluate the differences between proportions

cycles including 36 cycles of 1PN (cIVF 33 and ICSI 3 cycles). However, 1PN blastocysts were only transferred when there were no other embryos available. This population was included with the patients with multiple courses of F-TBT. The implantation rates and clinical pregnancy rates of blastocysts from 1PN in cIVF cycles (33.3 and 33.3 %) were not significantly different compared with controls for cIVF (39.0 and 35.9 %) and ICSI (46.7 and 41.1 %). In this study, 33 transfer cycles with 33 blastocysts derived from 1PN embryos in cIVF resulted in nine deliveries. However, although four blastocysts (3 cycles) from 1PN in ICSI were transferred, no implantation was observed. We also examined the neonatal data from 1PN blastocysts in cIVF compared with 2PN blastocysts in cIVF and ICSI (Table 4). There were no differences in the weeks of gestation and weight of the newborns at birth. Moreover, nine newborns from F-TBT with 1PN blastocyst were delivered and presented with no major malformations.

Discussion

We obtained normal healthy babies without malformation successfully from 1PN zygotes cultured until the blastocyst stage. The 1PN embryos in cIVF that did develop to blastocysts showed adequate potential for implantation and ongoing pregnancy as with 2PN blastocysts.

Several attempts to analyze human 1PN zygotes and embryos in cIVF and ICSI cycles have been reported, such as the mechanism of 1PN formation on fertilization [4, 6, 16–18]; chromosome constitution in zygote, embryo, and blastocyst stages [6, 9, 10, 12, 19–21]; and genetic composition of the pronucleus in human [11] and mouse [22]. According to these data, when the chromosomal status of 1PN embryos was analyzed using fluorescence in situ hybridization (FISH), the rate of diploid chromosome constitution in cIVF was significantly higher than that in ICSI [6, 9, 10]. Liao et al. reported

Table 4 Neonatal outcomes of 2PN and 1PN blastocysts in cIVF and ICSI cycles

Insemination	cIVF		ICSI
Pronuclear state	2PN	1PN	2PN
No. of deliveries	94	9	58
Weeks of gestation	38.2±2.7	38.9±1.5	38.0±3.0
Delivery (normal/Cesarean section)	37/51	4/5	21/37
Gender (females/males)	40/54	3/6	33/25
Average length (cm)	48.2±4.0	49.6±2.8	48.0±4.9
Average of the girth of the head (cm)	33.6±1.7	34.2±1.5	32.9±2.4
Birth weight (g)	2874.2±609.3	2999.0±484.4	2831.2±720.2
No. of major malformations at live birth (%)	1 (1.1)	0 (0.0)	2 (3.4)

Nine cases of neonatal outcomes of 1PN blastocysts were presented. Normal=vaginal delivery

that the diploid rate of the blastocysts derived from 1PN embryos (74.6 %) was significantly higher than that from the arrested cleavage-stage 1PN embryos (31.6 %) [9]. Otsu et al. also reported that all analyzed 1PN blastocysts in cIVF were diploid [20]. However, the diploid rate of ICSI 1PN blastocysts was 37.5 % [19]. van der Heijden et al. reported that 86.7 % of the 1PN zygotes included the maternal and paternal genome in cIVF and that the diploid rate of cIVF cycles was higher than that of ICSI cycles (30.3 %) using immunostaining based on histon modification [11]. Moreover, Mateo et al. reported that when 1PN embryos were cultured to blastocyst in ICSI, the blastocyst formation and good-quality blastocyst rates were only 14.8 and 7.4 %, respectively [19]. In addition, Reichman et al. reported that the implantation rate and pregnancy rates of the 1PN embryos from cIVF or ICSI were only 6.4 and 1.3 %, respectively [5]. Most of these studies have reported that the rates of diploid chromosome constitution in 1PN zygotes in cIVF were higher than ICSI, though poor embryo development and clinical outcomes were present in both cycles. Consequently, it has been proposed that the transfer of 1PN embryos should be avoided and discarded in human ART programs. However, the 1PN embryos were sometimes clinically transferred and the few reports regarding newborn outcome of 1PN zygotes were almost all small study and case reports.

In the present study, the incidence of abnormal zygotic stages with 1PN following cIVF and ICSI was similar to previous reports [4, 6]. Further, when 1PN embryos in cIVF and ICSI were cultured until day 6, the blastocyst formation rate was significantly reduced (Table 2), similar to previous reports [5]. In addition, although the rate of good-quality embryos from 1PN embryos in ICSI on day 3 did not significantly differ from 1PN embryos in cIVF, 1PN embryos in ICSI showed a significantly lower rate of development to blastocyst. This may be due to the fact that human 1PN zygotes in cIVF are formed by the enclosure of the juxtaposed male and female nuclei in a common pronuclear envelope [18]. In addition, sperm was able to enter ooplasm in human oocyte at any location [23]. Krukowska et al. demonstrated the fusion of female and male chromatin before nuclear envelope formation by injection of a sperm at the vicinity of the metaphase II spindle [22]. Thus, diploid single-nucleated zygotes are more common in cIVF than in ICSI. The diploid 1PN zygotes in ICSI may be derived from same origin in cIVF; however, the rate of haploid 1PN zygotes and embryos were higher than in cIVF [9, 11, 21]. This was because most of ICSI 1PN zygotes were parthenogenesis. In addition, the 1PN zygotes in ICSI included one developed prominent female PN and the sperm head which either decondensed and subsequently formed a small male PN of <5-μm diameter or did not decondense in cytoplasm [17]. Thus, ICSI 1PN zygotes poorly developed to blastocysts compared with cIVF 1PN. Therefore, all 1PN zygotes were cultured until the blastocyst stage and only fully developed or expanded blastocysts were cryopreserved and transferred. As a result, higher clinical pregnancy and ongoing pregnancy rates were observed in cIVF.

As our main goal of this study was the clinical and neonatal outcomes from 1PN embryos, we divided the F-TBT cycles of blastocysts into four groups (Table 3). Our data showed that most of the F-TBTs were performed on 2PN blastocysts in cIVF and ICSI because of the lower priority of the 1PN blastocysts. However, our results which in 1PN blastocysts in cIVF, and not in ICSI, showed a similar rate of clinical outcomes compared with 2PN blastocysts. In contrast, Staessen et al. [4] and Reichman et al. [5] demonstrated lower implantation and clinical pregnancy rates after the transfer of cIVF or ICSI cleavage-stage embryos on day 3. This suggests that it is difficult to select the embryos which have adequate potential for implantation on day 3.

The neonatal data of live babies had no significant difference in the birth weight and weeks of gestation between 1PN in cIVF and control groups (Table 4). Similar reports on neonatal data derived from 1PN embryos in cIVF and ICSI have been reported in the literature [4, 5, 7, 8]. Gras and Trounson [7] and Reichman et al. [5] have reported similar findings on normal birth weight, the weeks of gestation, and a birth of a healthy child. Staessen et al. [4] and Dasig et al. [8] also described the delivery of healthy babies without neonatal data. Previous studies regarding live birth babies derived from 1PN embryos have reported no major malformations.

In conclusion, we have shown that blastocysts derived from 1PN zygotes may lead to normal healthy newborns. We performed embryo transfer of only 1PN blastocysts after informed consent; 9 cycles led to pregnancy and continued to delivery, and no malformations were detected in the newborns. We have also indicated that it may be possible to expect selection of embryos with normal chromosomes and an adequate ongoing pregnancy rate after blastocyst transfer derived from 1PN zygotes in cIVF cycles. These results suggested that blastocysts derived from 1PN embryos in cIVF are available for clinical use and may lead to an increase in the chance of pregnancy in patients receiving ART with 1PN embryos that were considered unsuitable for transfer before. However, there were few cases to ensure the safety of transferring 1PN blastocysts in this report, so it will be necessary to increase the number of cases and to investigate the relationship between major malformations and 1PN.

Acknowledgments We thank the embryology staff of the Angel Bell Hospital for their assistance in preparing this manuscript and their expert technical help. We are also grateful to Dr. Koji Komatsu for critically reading this manuscript.

Ethics statement This study was approved by the Institutional Review Board of KISHOKAI Medical Corporation and was conducted in accordance with the principles expressed in the Declaration of Helsinki. Written informed consent for their treatment and for their outcomes to be described was obtained from all patients.

Conflict of interest The authors declare that they have no competing interests.

Authors' contributions FI conceived and designed the study, carried out the experiments, acquired the data, performed the statistical analysis, and drafted the manuscript. YA carried out the experiments, acquired the data, and helped draft the manuscript. MS carried out the experiments, acquired the data, and helped draft the manuscript. HH performed the statistical analysis and helped draft the manuscript. YM conceived the study, participated in its design and coordination, and helped draft the manuscript. All authors read and approved the final manuscript.

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