Embryos Arising from Apronuclear (0PN) and Unipronuclear (1PN) Have Similar Euploidy Rates with Those from 2PN and Should be Considered for Transfer

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ABSTRACT

Background: Fertilisation assessment is routinely made at 16–18 hours post-ICSI and 18–20 hours post-insemination. However, the absence of pronuclei (PN) during standard fertilisation assessment does not necessarily indicate fertilisation failure. The aim of this study is to assess the chromosomal status of blastocysts derived from 0PN and 1PN zygotes as well as to assess the clinical outcome after transfer of such embryos.

Methods: In this study, we use microarray comparative genomic hybridisation (MaCGH) or next generation sequencing (NGS) to analyse the chromosomal status of 271 blastocysts (204 from 2PN, 41 from 0PN, 26 from 1PN) obtained from 42 patients who underwent conventional IVF (cIVF) and ICSI cycles with preimplantation genetic testing for aneuploidy (PGT-A).

Results: Euploidy was confirmed in 126 (126/204; 61.8%), 31 (31/41; 75.6%) and 18 (18/26; 69.2%) 2PN-, 0PN- and 1PN-derived blastocysts respectively while the remaining 96 blastocysts displayed various chromosomal abnormalities. A Y-chromosome was observed in 0PN-derived blastocysts (19/41; 46.3%) and 1PN-derived blastocysts (13/26; 50%), indicating that sperm had penetrated the oocyte and not due to parthenogenetic activation. Four euploid 0PN-derived blastocysts were transferred to 4 patients and 3 healthy live births were achieved. Four euploid 1PN-derived blastocysts were transferred to 4 patients and 1 on-going pregnancy was achieved.

Conclusion(s): 0PN- and 1PN-derived zygotes can be chromosomally normal and result in healthy live births. Such zygotes should not be discarded but instead be subjected to extended culture with PGT-A to ascertain the chromosomal and ploidy status and be considered for transfer.

Keywords: Apronuclear; Unipronuclear; Ploidy; Preimplantation Genetic Testing; PGT.

INTRODUCTION

Fertilisation is routinely assessed 16–18 hours post-ICSI and 18–20 hours post-insemination (Montag and van der Ven, 2001). Normal fertilisation is determined by the presence of 2PN within the oocyte with defined membranes and 2 polar bodies in the perivitelline space (Campbell and Fishel, 2015).

Fertilisation is a process which involves the fusion of maternal and paternal gametes to initiate the formation of a new individual. The events that occur during fertilisation include: (a) sperm penetration; (b) sperm-zona pellucida interaction; (c) sperm-oocyte fusion; (d) oocyte activation; (e) sperm nucleus decondensation; (f) male and female PN formation; (g) pronuclear migration to the centre of the ooplasm; and (h) association of parental chromosomes on the spindle and first cleavage division (Plachot, 2000). The male PN is formed first and shortly followed by the female PN. The female PN then migrates towards the male PN until the 2 abut in the centre of the oocyte followed by PN breakdown permitting subsequent

cleavage. Formation of the male and female PN is commonly visible simultaneously, however a small percentage may form asynchronously (Yasuyuki et al., 2014).

The appearance of 2PN is important in an IVF cycle since the PN number is taken to reflect the ploidy status of a zygote. Two PN suggest diploidy, while 3 or more PN indicate polyploidy (Manor et al., 1996). The absence of PN (0PN) indicates failed fertilisation and 1PN suggests abnormal fertilisation (Feenan and Herbert, 2006). The current routine practice is that only embryos that showed normal fertilisation (2PN) are chosen for transfer. However, occasionally there are no 2PN embryos available for transfer. Some oocytes without evidence of fertilisation such as 0PN and 1PN continue to cleave and form morphologically normal blastocysts and are indistinguishable from 2PN embryos. Such 0PN and 1PN embryos have been the subject of investigation but generally, IVF centres are uncertain whether such embryos with normal morphology should be used for transfer.

The transfer of embryos deriving from 0PN and 1PN zygotes is rare but normal pregnancies as well as live births have been reported (Burney et al., 2008; Dasig et al., 2004; Gras and Trounson, 1999; Khoo et al., 2007; Lim et al., 2015; Lim et al., 2016; Manor et al., 1996; Noyes et al., 2008; Staessen et al., 1993). Analyses of chromosomal status in 0PN and 1PN embryos have been reported using fluorescence in situ hybridisation (FISH) on selected chromosome number mainly 13, 18, 21, X and Y (Khoo et al., 2007; Manor et al., 1996; Noyes, 2008; Staessen and van Steirteghem, 1997). We have previously reported complete screening of 23 sets of chromosomes in 0PN and 1PN embryos (Lee et al., 2013; Lee et al., 2015; Low et al., 2015).

Partly due to unavailability in most IVF centres for screening on all 23 chromosomes on 0PN and 1PN embryos, the chromosomal status of these embryos remains questionable and therefore are not considered for transfer. In this study, we screened all 23 sets of chromosomes in 0PN- and 1PN-derived blastocysts in patients who underwent conventional IVF (cIVF) and ICSI cycles with PGT-A.

MATERIALS AND METHODS

Patients and cycles

A total of 42 patients undergoing IVF treatments (including cIVF, ICSI and combined conventional IVF and ICSI) with PGT-A who had either 0PN- and 1PN-derived blastocysts were included in this study between November 2013 and November 2016. Cases with known altered karyotype of inversion and translocation; and indeterminate biopsies were excluded from this study. These 42 patients underwent controlled ovarian hyperstimulation with GnRH agonist or GnRH antagonist protocol. Ovulation was triggered by human chorionic gonadotrophin (hCG) when at least one follicle was ≥ 20 mm in diameter. Oocyte retrieval was performed 36.5–37.0 hours later under ultrasound-guided transvaginal aspiration.

Laboratory

For conventional IVF (cIVF), oocytes were inseminated 3–4 hours after oocyte retrieval. Patient's husband's spermatozoa were processed by a standard density gradient centrifugation and swim-up method with 250,000 motile spermatozoa loaded into each insemination well containing 5 cumulus-oocyte-complexes (COC). The insemination dish was incubated for at least 2 hours. After 2 hours, the oocytes were individually cultured in a day 0 dish; fertilisation assessment was done the following day between 18 and 20 hours post-insemination. For ICSI, cumulus cells from the oocytes were removed by denudation 2 hours after oocyte retrieval and ICSI (PIEZO-ICSI, Primetech, Japan) was performed as described elsewhere (Yap et al., 2015). Fertilisation was assessed the following day between 16 and 18 hours post-injection. In combined cIVF and ICSI cycles, half of the retrieved oocytes were subjected to cIVF and the other half were subjected to ICSI. After fertilisation assessment, 0PN- and 1PN-derived zygotes were divided according to its respective PN status and were sequentially cultured to blastocyst stage.

Biopsy and PGS

Assisted hatching was done on all cleaved 2PN-, 0PN- and 1PN-derived zygotes on day 3. Blastocysts with at least a 3BB grade (Gardner's System) (Schoolcraft et al., 1999) were selected for trophectoderm (TE) cell biopsy on day 5 and/or 6. Three to 5 TE cells were dissected from each blastocyst using laser ablation with a non-contact Class I laser diode of 400 μ s pulses (ZILOS-tk, Hamilton Thorne, USA) (Yang et al., 2012). Harvested TE cells were loaded into PCR tubes with 2.5 μ l of phosphate buffered saline (1 $\times$ PBS) (Yang et al., 2011). All biopsied blastocysts were vitrified on

either day 5 or day 6, depending on the day of biopsy using Cryotec Method (Cryotech, Japan).

The biopsied cells of each blastocyst were screened using microarray comparative genomic hybridisation (MaCGH) or next generation sequencing (NGS) to determine the chromosomal status of each blastocyst. For MaCGH, the biopsied cells were amplified, labelled, hybridised and analysed according to the manufacturer's protocol (BlueGnome, United Kingdom). For NGS, the biopsied cells were amplified, labelled and sequenced according to the manufacturer's protocol (Thermo Fisher, USA).

Statistical analysis

Comparison groups were established to assess whether the formation of morphologically normal blastocysts derived from 0PN and 1PN zygote exhibit similar euploidy and aneuploidy rates when compared with blastocysts derived from 2PN embryos in the cIVF and ICSI group. Statistical analysis was done using two-tailed Fisher's exact test. Statistical significance was defined as a $p < 0.05$.

Frozen-thawed blastocyst transfer cycle (FBT)

Hormone replacement treatment protocol was used in all FBT cycles. The proliferative phase was induced by taking oestrogen tablets (Progynova) for 13–15 days. After confirming the lining of the endometrium has developed sufficiently, daily administration of progesterone was initiated, and blastocyst transfer was performed on 5.5 day of progesterone administration. Warming of the blastocysts were performed using the Cryotec Method (Cryotech, Japan). Both oestrogen and progesterone were continued until the day of pregnancy test. Serum beta hCG was assessed 9–14 days after the day of embryo transfer. If tested positive, oestrogen and progesterone were continued for several weeks depending on physician's protocol. Pregnancy and number of gestational sacs were confirmed using transvaginal ultrasound.

RESULTS

A summary describing the IVF cycles that resulted in 0PN- and 1PN-derived blastocysts were outlined in Table 1. The mean maternal age of the 42 patients was 31.7 years old. The number of oocytes retrieved and injected/inseminated was 943 and 797 respectively. A total of 271 blastocysts were biopsied for PGT, of which 204 blastocysts were derived from 2PN, 41 derived from 0PN and 26 derived from 1PN respectively. One hundred and twenty-six 2PN-derived blastocysts (61.8%), 31 0PN-derived blastocysts (75.6%) and 18 1PN-derived blastocysts (69.2%) were tested to be euploid. The euploidy and aneuploidy rates were comparable between 2PN-, 0PN- and 1PN-derived blastocysts ($p > 0.05$).

Of the 0PN- and 1PN-derived blastocysts, 19 (19/41; 46.3%) 0PN-blastocysts and 13 1PN-blastocysts (13/26; 50%) displayed a Y-chromosome signal, indicating that sperm had penetrated the

Table 1. Summary of treatment cycles.

	2PN	0PN	1PN	>2PN
No. of zygotes (%)	474 (59.5)	201 (25.2)	51 (6.4)	28 (3.5)
No. of blastocysts biopsied	204	41	26	—
No. of euploid (%)	126 (61.8)	31 (75.6)	18 (69.2)	—
No. of aneuploid (%)	78 (38.2)	10 (24.4)	8 (30.8)	—
No. of single aneuploidy (%)	50 (24.5)	5 (12.2)	7 (26.9)	—
No. of multiple chromosomal abnormalities (%)	28 (13.7)	5 (12.2)	1 (3.8)	—

Table 2. Summary of sex chromosome abnormalities.

Sex chromosome	OPN	1PN	Total
Normal XX (%)	21 (52.2)	12 (46.2)	33 (49.3)
Normal XY (%)	16 (39.0)	13 (48.1)	29 (43.3)
XO (%)	1 (2.4)	0	1 (1.5)
OY (%)	0	1 (3.8)	1 (1.5)
XXY (%)	3 (7.3)	0	3 (4.5)
Displayed Y (%)	19 (46.3)	13 (50.0)	32 (47.8)

oocyte and not due to parthenogenetic activation (Table 2). Of these, 16 out of the 19 OPN- and all 13 1PN-Y bearing blastocysts were euploid. One OPN-derived blastocyst (XO) and one 1PN-derived blastocyst (OY) were haploid; 7.3% (3/41) of the OPN-derived blastocysts were triploid (XXY). No triploid was seen in 1PN-derived blastocyst group.

Tables 3 and 4 outlined the PGT-A results and outcome measures for all OPN- and 1PN-derived blastocysts. From the 49 OPN- and 1PN-derived blastocysts that were tested to be euploid, 11 of which were transferred to 11 patients: 4 patients had OPN single blastocyst transfer (SBT), 4 patients had 1PN SBT, 2 patients had combined 2PN + OPN double blastocysts transfer (DBT) and 1 patient had combined 2PN + 1PN DBT. No prenatal diagnosis was performed.

Three healthy live births and 1 on-going pregnancy (24 + 5 at the time of writing) resulted from 4 OPN SBT and 4 1PN SBT respectively. All 4 pregnancies achieved were singleton.

Two live births resulted from 2PN + OPN DBT and both were singleton pregnancies. Therefore, we cannot confirm the PN origin of the foetus in these 2 live births.

DISCUSSION

Fertilisation assessment is routinely made at 16–18 hours post-ICSI and 18–20 hours post-cIVF, but 2PN can be detected as early as 5 hours post-insemination (Trounson and Osborn, 1993). Nagy et al. (1998) revealed that the appearance of 2PN was observed 6 hours post-ICSI and 8 hours post-IVF using ejaculated spermatozoa on human sibling oocytes. A preliminary study by Payne et al. (1997) described how normal fertilisation took place and that the timing of fertilisation events varied between oocytes. This time lapse study also showed male and female PNs were visible as early as 2 hours and 51 minutes post-ICSI and 63% (20/32 normally fertilised oocytes) of the male and female PNs become visible simultaneously, with a further 25% (8/32 normally fertilised oocytes) becoming visible asynchronously within 30 minutes of each other. Another time lapse study (Yasuyuki et al., 2014) also demonstrated that the human male PN and female PN were formed as early as 6.6 hours and 6.8 hours respectively.

The absence of PN during standard fertilisation assessment does not necessarily indicate fertilisation failure (Elder and Dale, 2000). The observation that 20% of OPN zygotes can progress to cleavage suggests either that observation of the PN can be missed due to syngamy before fertilisation check or that the PN only formed after the fertilisation check (Feenan and Herbert, 2006). Manor et al. showed 57% of OPN embryos were normal diploid, 30% polyploid and/or mosaic and 13% aneuploid following fluorescent in situ hybridisation (FISH) assay using X, Y, 13, 18 and 21 probes and the transfer of OPN embryos had resulted in one on-going pregnancy and a normal female foetus (46, XX) was documented

Table 3. PGS results and outcome measures of OPN-derived blastocysts.

No.	Blastocyst stage	PGT Results	Transferred	Outcome
1	5AA (IVF)	46 XY	Frozen	—
2	5AA (ICSI)	46 XX	Frozen	—
3	5AA (ICSI)	XX, -22	No	—
4	6AA (ICSI)	45 XO	No	—
5	5AA (ICSI)	47 XXY	No	—
6	6BA (ICSI)	46 XX	Frozen	—
7	5BB (ICSI)	46 XY	Yes	DBT, LB-S
8	5AA (ICSI)	46 XX	Frozen	—
9	5BA (ICSI)	46 XY	Frozen	—
10	5BA (IVF)	XX, -2, +13, +17, +18, +20, -22	No	—
11	5AA (ICSI)	46 XX	Frozen	—
12	5BA (IVF)	46 XX	Frozen	—
13	4AA (ICSI)	46 XX	Frozen	—
14	5AA (ICSI)	47 XXY, -3	No	—
15	5AA (IVF)	XX, +22	No	—
16	3AA (IVF)	46 XX	Frozen	—
17	6BB (ICSI)	XX, +13, +16	No	—
18	5BB (ICSI)	46 XY	Frozen	—
19	5BA (ICSI)	46 XX	Frozen	—
20	6AA (ICSI)	47 XXY, -11, +12, +13, +19	No	—
21	5AA (RESCUE-ICSI)	46 XX	Frozen	—
22	5AA (RESCUE-ICSI)	46 XY	Frozen	—
23	6BA (ICSI)	46 XX	Frozen	—
24	6AA (IVF)	46 XY	Yes	SBT, LB-S
25	4AB (IVF)	46 XY	Yes	DBT, LB-S
26	5BB (IVF)	46 XY	Frozen	—
27	5BB (ICSI)	46 XY	Frozen	—
28	5BA (ICSI)	46 XY	Yes	SBT, Negative
29	6BB (ICSI)	XY, -7, +15	No	—
30	5AA (ICSI)	46 XY	Yes	SBT, LB-S
31	5BB (IVF)	46 XX	Frozen	—
32	6AA (ICSI)	46 XX	Frozen	—
33	6AA (IVF)	46 XX	Frozen	—
34	5AA (IVF)	46 XY	Frozen	—
35	5AB (ICSI)	46 XX	Frozen	—
36	5AA (ICSI)	XX, +8	No	—
37	6AA (ICSI)	46 XX	Frozen	—
38	5AA (ICSI)	46 XY	Frozen	—
39	5AB (ICSI)	46 XY	Frozen	—
40	5AB (ICSI)	46 XX	Yes	SBT, LB-S
41	5AB (ICSI)	46 XY	Frozen	—

SBT: Single blastocyst transfer.

DBT: Double blastocyst transfer with 2PN-blastocyst.

LB-S: Live birth singleton.

by amniocentesis (Manor et al., 1996). Consistent with this, Khoo et al. reported a successful on-going pregnancy after the transfer of a seven-cell embryo derived from a OPN zygote, following FISH analysis (13, 18, 21, X and Y). The chromosomal status was further confirmed by amniocentesis displaying a karyotype of 46XY (Khoo et al., 2007). Burney et al. (2008) also reported the birth of a healthy infant originating from a OPN zygote.

Unipronuclear zygotes obtained following cIVF or ICSI may be due to oocyte parthenogenetic activation, possible fusion of male

Table 4. Chromosomal status and outcome measures of 1PN-derived blastocysts.

No.	Blastocyst stage	PGT Results	Transferred	Outcome
1	5AA (IVF)	46 XX	Frozen	—
2	3AB (IVF)	XY, +4	No	—
3	5AA (IVF)	46 XX	Frozen	—
4	5AB (IVF)	XY, +5	No	—
5	4AB (IVF)	46 XY	Frozen	—
6	4AB (IVF)	46 XY	Frozen	—
7	5AB (IVF)	XY, +3	No	—
8	5AB (IVF)	XX, +11	No	—
9	5AB (IVF)	XY, +21	No	—
10	6AB (IVF)	46 XX	Frozen	—
11	5AA (ICSI)	XX, +16	No	—
12	5AB (IVF)	46 XX	Frozen	—
13	5AB (ICSI)	46 XX	Frozen	—
14	5AB (ICSI)	46 XX	Frozen	—
15	5AA (ICSI)	46 XY	Frozen	—
16	5AB (ICSI)	46 XY	Frozen	—
17	6AB (ICSI)	46 XX	Frozen	—
18	5AB (ICSI)	46 XX	Yes	SBT, Negative
19	5AB (ICSI)	46 XX	Yes	SBT, Negative
20	5BA (ICSI)	46 XY	Frozen	—
21	5BA (ICSI)	45 OY	No	—
22	6BB (ICSI)	46 XX	Frozen	—
23	5AB (ICSI)	XY, +7, +9, +14, +16, +19, +20, -22	No	—
24	6AA (ICSI)	46 XY	Yes	DBT, Negative
25	5AB (ICSI)	46 XY	Yes	SBT, on-going
26	5AB (ICSI)	46 XY	Yes	SBT, Negative

and female PN or asynchronous PN formation (Gras and Trounson, 1999). The development of parthenogenetic oocytes is impaired after activation, thus leading to early embryo arrest usually at the 8-cell stage (Winston et al., 1991) and failure to activate the embryonic genome for further embryonic development. Chromosomal studies revealed that 45–48% of diploid 1PN zygotes are in fact fertilised displaying Y signals and were not due to parthenogenetic activation. In a study by Plachot and Crozet (1992), it was observed that 77% of 1PN oocytes develop past early cell divisions, of these, 14.3% are able to form morphologically normal blastocysts and are indistinguishable from 2PN embryos suggesting that these 1PN oocytes may in fact be derived from fertilised oocytes with a diploid complement. It was suggested that such embryos may be the product of 2PN sharing the same PN envelope after the fusion of male and female PN (Tessarik and Mendoza, 1996). Asynchronous formation of PN was also observed in 25% of 1PN zygotes, where such zygotes develop the second PN when reassessed 4 hours after the initial fertilisation assessment (Staessen et al., 1993). Subsequently, Payne et al. (1997) affirmed this theory finding 38% (12/32) of fertilised oocytes developed PN asynchronously with the male PN appearing before the female PN, with the average time between the appearance of the two PN being 31 minutes. By extending *in-vitro* culture to day 5 or 6, morphologically normal blastocysts may possibly be used as an indicator of fertilisation and may be selected for transfer when insufficient 2PN embryos are available (Gras and Trounson, 1999).

A study by Noyes (2008) demonstrated that at 18 hours post-insemination, the zygote distribution was 19% 0PN, 4% 1PN and 69% 2PN and 8% 3PN out of 1908 embryos assessed. From these, the euploid karyotypes for 0PN, 1PN and 2PN were 3%, 5% and 19% respectively following FISH analysis (X, Y, 13, 15, 16, 17, 18, 21 and 22) ($p = 0.015$ for 1PN vs. 2PN; no statistical significance for 0PN vs. 2PN). In 2013, Lee et al. (2013) at Alpha Fertility Centre showed that 0PN and 1PN embryos following ICSI that develop to the cleavage stage have a euploidy rate similar to embryos resulting from 2PN after microarray comparative genomic hybridisation (MaCGH). Again, Lee et al. (2015) reported 273 blastocysts following cIVF were analysed using MaCGH and from these, 68.8% of 0PN blastocysts, 52.9% of 1PN blastocysts and 51.7% 2PN blastocysts were euploid. In the same year, Low et al. (2015) from Alpha Fertility Centre also demonstrated comparable euploidy rates ($p > 0.05$) among 0PN, 1PN and 2PN day 3 embryos following cIVF using MaCGH.

Since 49 0PN- and 1PN-derived blastocysts (49 out of 67) in our study were found to be euploid, our results document the possibility that accelerated disappearance of the PN membrane or asynchronous PN formation following either cIVF or ICSI, do not necessarily reflect aneuploidy or failed fertilisation. This study also suggests that if 0PN and 1PN zygotes that can form morphologically normal blastocysts do not necessarily reflect abnormal embryo development and can be euploid. On the other hand, the pregnancies achieved by transferring 0PN- and 1PN-derived blastocysts establishes the ability of such blastocysts to implant.

0PN and 1PN transfers are controversial and many IVF centres prefer not to transfer such embryos and discard them based on the universal fertilisation assessment timings. Thus, some patients, especially patients with decreased ovarian reserve or older patients may miss the chance for pregnancy when there is a lack of normal 2PN embryos for transfer.

Although blastocyst culture facilitates the selection of morphologically normal 0PN and 1PN for transfer, clinics may want to impose policies requiring PGT-A to screen 0PN and 1PN derived embryos to determine their chromosome constitution prior to transfer. In addition to that, time-lapse monitoring can be used to allow a detailed observation of second polar body extrusion and pronuclear formation. Transferring of 0PN and 1PN embryos should only be considered for transfer especially where there is a lack of 2PN-derived embryos. On-going pregnancies resulting from 0PN or 1PN euploid blastocyst transfers should be followed by non-invasive prenatal testing (NIPT) at 9–10 weeks and by prenatal diagnosis such as chorionic villus sampling (CVS) at 11–12 weeks or amniocentesis at 15–16 weeks to confirm a chromosomally normal foetus. Also, patients should be counselled appropriately, and informed decisions made about the safety of transferring these embryos.

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