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# Reassessing the conventional fertilization check: leveraging preimplantation genetic testing for aneuploidy to increase the number of transferrable embryos

## BIOGRAPHY

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## KEY MESSAGE

Preimplantation genetic testing for aneuploidy reveals that a considerable number of non-2PN zygotes are diploid and euploid, challenging traditional assumptions about their viability. Embryos previously classified as being aberrantly fertilized can develop into blastocysts and may lead to successful live births, thereby increasing the number of embryos available for transfer.

## ABSTRACT

**Research question:** Are some non-2PN zygotes normally fertilized, euploid and thus available for transfer?

**Design:** Retrospective cohort study of patient data from 1214 PGT-A cycles from a single private IVF clinic. A total of 152 non-2PN embryos were assessed by PGT-A with parent of origin assessment, and were compared with 4822 2PN embryos assessed by PGT-A. Transfer outcomes of euploid embryos were then compared.

**Results:** Over 40% of the tested non-2PN embryos were euploid. The 152 non-2PN (89 ICSI, 63 IVF), embryos were 2×0PN, 50×1PN, 24×2.1PN, 65×3PN, 9×4PN and 2×5PN. Of 4822 2PN embryos, 4737 (98.2%) were diploid, 1.4% polyploid and 0.4% haploid. Embryos resulting from 1PN zygotes were more likely to be haploid, and embryos resulting from 3PN and 4PN zygotes were more likely to be polyploid ($P < 0.001$). A total of 44 out of 89 (50%) ICSI embryos were either haploid or polyploid, significantly higher than those derived from IVF (16/63 [25.4%], $P = 0.004$). Maternal origin of polyploidy was more common in ICSI cases ($P = 0.005$). A total of 42% of the 93 diploid non-2PN embryos were also euploid, with diploid blastocysts from non-2PN zygotes likely to be euploid as those from diploid 2PN zygotes ($P = 0.10$). Twelve euploid 1PN, 2.1PN and 3PN embryos were transferred; two ongoing pregnancies and four live births have been reported.

**Conclusions:** This approach extends the use of PGT-A in identifying more embryos available for transfer, has the potential to increase cumulative pregnancy rates and questions the value of the fertilization check.

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## KEYWORDS

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## INTRODUCTION

Despite decades of innovation, the principal limiting factor in IVF and intracytoplasmic sperm injection (ICSI) continues to be the availability of sufficient numbers of suitable embryos for transfer (*Capalbo et al., 2017*; *Vaegter et al., 2017*; *McLernon and Bhattacharya, 2023*). This is especially pertinent for poor prognosis patients, in whom the most cited risk factor is advanced maternal age, and for low responders to ovarian stimulation. Hence, although oocyte number is arguably the most significant predictor of cumulative live birth rate (*McLernon and Bhattacharya, 2023*), normal fertilization is also essential and, accordingly, embryologists carry out a 'fertilization check' before continued culture. This conventionally happens between 16 and 18 h after insemination (in both IVF and ICSI), and fertilization is deemed to be either successful, or not, by zygote morphology alone. At this check, the visible extrusion of the second polar body and the presence of two pronuclei (2PN) are the sole determinants of whether an embryo goes forward for continued culture and, thereafter, possible transfer (*Capalbo et al., 2017*).

Abnormal fertilization may result in zygotes that are morphologically apronuclear (0PN); have only one pronucleus (1PN); or are polypronuclear, having three, four or five pronuclei (3PN, 4PN, 5PN respectively). A final commonly observed phenomenon is a zygote that has two normally sized pronuclei, plus a smaller pronucleus (a micronucleus); these are termed '2.1PN' embryos. All of these abnormal types of fertilization are observed in at least 10% of the zygotes produced by IVF or ICSI (according to *Capalbo et al., 2017*), although most clinics report 2PN rates of 65–80% per mature oocyte (*Papale et al., 2012*; *ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017*). It is, however, well established that zygotes assessed to be 2PN (diploid) may not be euploid (nor indeed actually diploid) when genetically tested, with this a key rationale for PGT-A (*Griffin and Oğur, 2018*; *Victor et al., 2020*).

Because of the unconfirmed fertilization (and ploidy) status of non-2PN zygotes, and the potential risks that their transfer may carry, such as miscarriage or molar pregnancy, abnormally fertilized embryos are not usually considered for clinical use and are routinely discarded. It has, however, been shown that, if left in culture, some of these zygotes can continue their development to the blastocyst stage, and that a proportion have the potential to result in live births (*Capalbo et al., 2017*; *Kemper et al., 2023*). As evidence suggests that the incidence of 1PN oocytes is 1–8% and of 3PN oocytes is 1–7% (*Papale et al., 2012*; *ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017*), the whole assisted reproductive technology (ART) community may be unnecessarily wasting viable embryos if a significant proportion of these are in fact diploid (and euploid).

Historically, all 3PN zygotes have been assumed to be triploid, and hence were not cultured further (*Staessen and Van Steirteghem, 1997*). It has, however, since been shown that some 3PN zygotes are actually diploid and that, when genetically tested, most of these are also euploid (*Capalbo et al. 2017*; *Mutia et al., 2019*; *Mizuta et al., 2023*). Similarly, although 1PN zygotes are usually haploid, the formation of morphologically normal blastocysts from these has been demonstrated since the early 1990s (*Plachot and Crozet, 1992*). A possible cause for this is asynchrony of pronuclear formation. In some instances, a second pronucleus forms between 4 and 6 h after the fertilization check (*Staessen et al., 1993*). Because of the asynchrony of the formation of the nuclear envelope, 1PN morphology may also arise, resulting in either the failure to form the pronuclei envelope around one of the genomes, or that two prenatal genomes are packed into a single envelope (*Capalbo et al., 2017*). In the late 1990s, the first evidence that blastocysts derived from 1PN zygotes could be successfully transferred and ultimately result in live births emerged (*Staessen and Van Steirteghem, 1997*; *Barak et al., 1998*; *Gras and Trounson, 1999*), and this has been confirmed since (*Gras and Trounson, 1999*; *Dasig et al., 2004*; *Reichman et al., 2010*; *Itoi et al., 2015*; *Bradley et al., 2017*; *Capalbo et al., 2017*; *Xie et al., 2018*; *Si et al., 2019*; *Li et al., 2020*; *Gu et al., 2021*; *Henry et al., 2023*; *Ussher et al., 2023*).

The 2.1PN embryos are characterized by the presence of a small pronucleus alongside two normal pronuclei (*Capalbo et al., 2017*). These are not routinely considered for treatment owing to the potential polyploidy risk involving extra genetic material. Evidence now shows that these too can be successfully cultured to the blastocyst stage. For example, *Takahashi et al. (2022)* identified 15 blastocysts that developed from 2.1PN zygotes. Although all of these were found to be diploid, they were predominantly aneuploid. *Canon et al. (2023)* subsequently reported a live birth and an ongoing pregnancy after the transfer of blastocysts that resulted from 2.1PN zygotes. The investigators suggest that this type of embryo may result from failed cytokinesis, with some of one of the parental nuclei being driven into another envelope after fertilization. Moreover, although other types of abnormal fertilization events, e.g. 0PN, 4PN and 5PN, are far less common than those discussed above, live births have nonetheless also been achieved after the transfer of both 0PN (*Liu et al., 2016*; *Destouni et al., 2018*; *Paz et al., 2020*; *Li et al., 2021*) and 4PN-derived embryos (*Bredbacka et al., 2023*).

To the best of our knowledge, no study has yet evaluated the proportion of different types of non-2PN embryos that are both diploid and euploid using PGT-A and the outcomes of the transfer of those that are deemed to be chromosomally normal. The aim of the present study was, therefore, to establish the chromosomal constitution (including parent of origin) of a large cohort of zygotes that had been classified as abnormally fertilized through conventional fertilization checks. In so doing, it was confirmed if normal fertilization had indeed occurred (despite evidence to the contrary) and an assessment was made whether these embryos had potential for future transfer. The findings presented here are significant as they provide evidence to suggest that the applicability of PGT-A could be extended from an IVF embryo screening tool, to one that identifies embryos to be suitable for transfer that would not normally be considered.

## MATERIALS AND METHODS

The present study represents a retrospective cohort analysis using data derived from 1214 PGT-A cycles undertaken in a single UK centre between April 2020 and January 2024.

Fertilization of retrieved oocytes was carried out using either IVF or ICSI. The latter was proposed for patients who had

male factor infertility or previous IVF cycles with failed fertilization. A fertilization check was undertaken between 16 and 18 h after insemination. All embryos, whether resulting from normal or abnormal fertilization, were cultured to the blastocyst stage to maximize the number of embryos available for PGT-A. Embryos were incubated in an Embryoscope (Vitrolife, Viby, Denmark) time lapse system. Preimplantation genetic testing for aneuploidy was indicated for individuals with a history of miscarriage, unsuccessful implantation during an ART cycle or advanced maternal age. Parental swabs were provided for genotype analysis to confirm the parent of origin and ploidy status of the 152 embryos that showed deviations from 2PN (non-2PN), plus a further 4822 embryos that were 2PN for comparative purposes. The use of patient data for this analysis was approved by the University of Kent Research Ethics Advisory Group, approval number (CREAG114-07-23, approval date 19 December 2023). Patients who used donor gametes were excluded from the present study.

### Assisted reproductive cycles

All women underwent ovarian stimulation of multiple follicle development using a regimen of recombinant FSH, urinary FSH or a combination of both, accompanied by gonadotrophin-releasing hormone (GnRH) antagonist to suppress premature luteinization. Oocyte retrieval was scheduled for 36–38 h after the administration of Ovitrelle (Merck Serono Ltd., Feltham, UK), a HCG trigger injection. Patients were assigned to either conventional IVF or ICSI according to semen parameters and their clinical history. Spermatozoa were prepared using a density gradient (Vitrolife, Frölunda, Sweden). For IVF, co-incubation with spermatozoa occurred 2 h after oocyte retrieval. For ICSI, oocytes were incubated for a minimum of 2 h before being denuded using hyaluronidase media. A single spermatozoon was selected based on morphology and was immobilized before injection. All embryos were cultured in continuous single culture medium (Vitrolife, Frölunda, Sweden) pre-equilibrated the day before to 37°C in an atmosphere of 6% $CO_2$. For both IVF and ICSI cases, fertilization was assessed 16–18 h after insemination, with normal fertilization being confirmed by the presence of two polar bodies and 2PN (*Ahuja et al., 1985*). Zygotes that fertilized abnormally were categorized as apronuclear (0PN), monopronuclear (1PN), or polypronuclear, specifically, tri-pronuclear (3PN), tetrapronuclear (4PN), pentapronuclear (5PN) or 2.1PN (micro 3PN) as defined above. Embryos were subsequently assessed on days 5, 6 and 7, and those that developed to the blastocyst stage were graded in accordance with the ACE/NEQAS embryo grading guidelines (*Balaban et al., 2011*). To ascertain the ploidy status of the blastocysts, on either day 5 or day 6 of culture, trophectoderm biopsy was carried out, with five to seven trophectoderm cells being removed and subsequently vitrified (*Thornhill et al., 2012*; *Gorodeckaja et al., 2020*).

### Aneuploidy parent of origin testing

To determine the parental origin of any chromosomal abnormalities in non-2PN embryos, parents provided buccal swabs, collected using DNA Genotek OCD-100 buccal swab kits provided by CooperSurgical, Inc. (Livingston, NJ, USA). DNA extraction and analysis was undertaken on these samples, and PGT-A was carried out by CooperSurgical, Inc. (Livingston, NJ, USA) using the PGT-CompleteSM Test. Detection of genome-wide copy number variation (CNV) in non-2PN embryos, and the parental origin of these, were validated using control families with confirmed trio (parental/embryonic) relationships. Briefly, genomic DNA was isolated from maternal and paternal buccal swabs and processed with corresponding embryos. Next-generation sequencing was used to collect low coverage sequence data across the respective genomes. The identification of CNVs and single nucleotide polymorphisms (SNPs) was carried out in parallel. An initial karyotype was determined from CNV analysis of normalized read counts across all chromosomes. The ratio of sex chromosomes was used to identify potential male triploids (69;XXY) and all female euploids (46;XX) were further analysed. Genome-wide SNPs were filtered for depth and quality, and samples with greater than 200 SNPs included in validation. Identification of informative heterozygous SNPs and analysis of the allele ratios was used to determine pronuclear status: euploids displaying an expected B allele frequency of 1:1, triploids 2:1, and haploids 1:0. Overlapping SNP data across the embryonic and parental samples was used to determine the inherited haplotypes and confirm maternal and paternal contribution. Euploid embryos (46;XX/XY) displayed balanced parental contribution in autosomes, whereas triploid (69;XXX/XXY) and haploid (23;X) embryos displayed the expected genome-wide imbalance. Confirmed cases from each group were used to construct a statistical model to predict non-2PN status of embryos.

### Embryo vitrification and warming procedures

Blastocysts were vitrified using the Irvine Scientific® protocol after double-witnessing. Embryos were equilibrated in a M-199 HEPES buffered medium with gentamicin (7.5% dimethyl sulfoxide, 7.5% ethylene glycol, 20% dextran serum supplement [DSS]) for 12 min in a pre-labelled dish, before being moved to vitrification solution. The vitrification solution was again M-199 HEPES buffered medium with gentamicin, but this time containing 15% DMSO, 15% ethylene glycol, 0.5 M sucrose, 20% DSS. Cryotop® devices (Kitazato BioPharma Co. Ltd. Fuji, Shizuoka, Japan) were loaded with embryos and minimal freezing media and were then plunged into liquid nitrogen (−196°C). On the morning of embryo transfer, embryos were warmed by submerging the Cryotop® device in 37°C pre-equilibrated warming solution (Irvine Scientific, Santa Ana, CA, USA). The embryos were then kept in warming solution of M-199 HEPES buffered medium with gentamicin (1 M sucrose, 20% DSS) for 1 min, a solution that contained one-half the concentration of sucrose (0.5 M) for 3 mins, and then a washing solution that contained no sucrose for 5 mins before being moved to pre-equilibrated embryo culture media (Vitrolife, Gothenburg, Sweden) and incubated in an Embryoscope incubator (Vitrolife, Gothenburg, Sweden). Two hours after warming, blastocyst re-expansion was assessed.

### Embryo transfer and the establishment of pregnancy

When embryos that were initially classified as abnormal were found to be euploid, 2PN embryos were given priority for transfer. If no 2PN embryos were available, then euploid non-2PN embryos were considered for transfer. Endometrial preparation involved hormonal replacement therapy in 96% of the frozen embryo transfer cycles, whereas the remaining cycles were conducted naturally. Cycles were initiated with the administration of 6–10 mg of oestradiol valerate (Progynova) (Bayer, Leverkuse, Germany), once the endometrial thickness reached the optimal range of 6.5–8 mm.

Cyclogest 400 mg (LD Collins and Co Ltd., London, UK) three times daily was provided for luteal support. On the morning of the embryo transfer, after obtaining consent from the patients, the selected embryos were warmed, with the embryo transfer procedure carried out within a window ranging from 2–5 h after the warming procedure using an ultrasound-guided technique. Luteal support was provided until pregnancy was verified by a blood test with an HCG level exceeding 100 mIU/ml. For those who were pregnant, this support continued for at least 8 weeks. An ultrasound was conducted between 6 and 7 weeks of gestation to confirm a viable intrauterine pregnancy. The miscarriage rate was defined as the occurrence of spontaneous loss before 6 weeks in all confirmed positive pregnancies.

### Data analysis

All data were analysed in R version 4.2.2 (*R Core Team, 2022*), using RStudio (*RStudio Team, 2020*). Proportion data were compared by Fisher's exact tests, with simulated *P*-values used for comparisons of more than two groups. Where appropriate, post-hoc testing was undertaken via Bonferroni-corrected pairwise comparisons to embryos derived from 2PN zygotes.

## RESULTS

Within the study period, 152 zygotes were identified at the fertilization check stage as showing deviations from 2PN. Of these, two were 0PN, 50 were 1PN, 24 were 2.1PN, 65 were 3PN, nine were 4PN and two were 5PN (for representative images of these zygotes and the resulting blastocysts, see FIGURE 1). Summary demographic data and the numbers of normal and abnormally fertilized oocytes are presented in Supplementary Table 1. The rates of second polar body extrusion were greater in the 1PN and 2.1PN zygotes than those seen in the 3PN and 4PN zygotes (94.0% and 91.7% for 1PN and 2.1PN respectively, and 76.9% and 55.6% for 3PN and 4PN respectively) (Supplementary Table 2). Overall rates of second polar body extrusion were also observed greater for euploid embryos when compared with their non-euploid counterparts (94.9% and 78.8% for euploid and non-euploid, respectively). Given the low numbers of 0PN and 5PN zygotes, polar body information in these groups cannot be meaningfully compared.

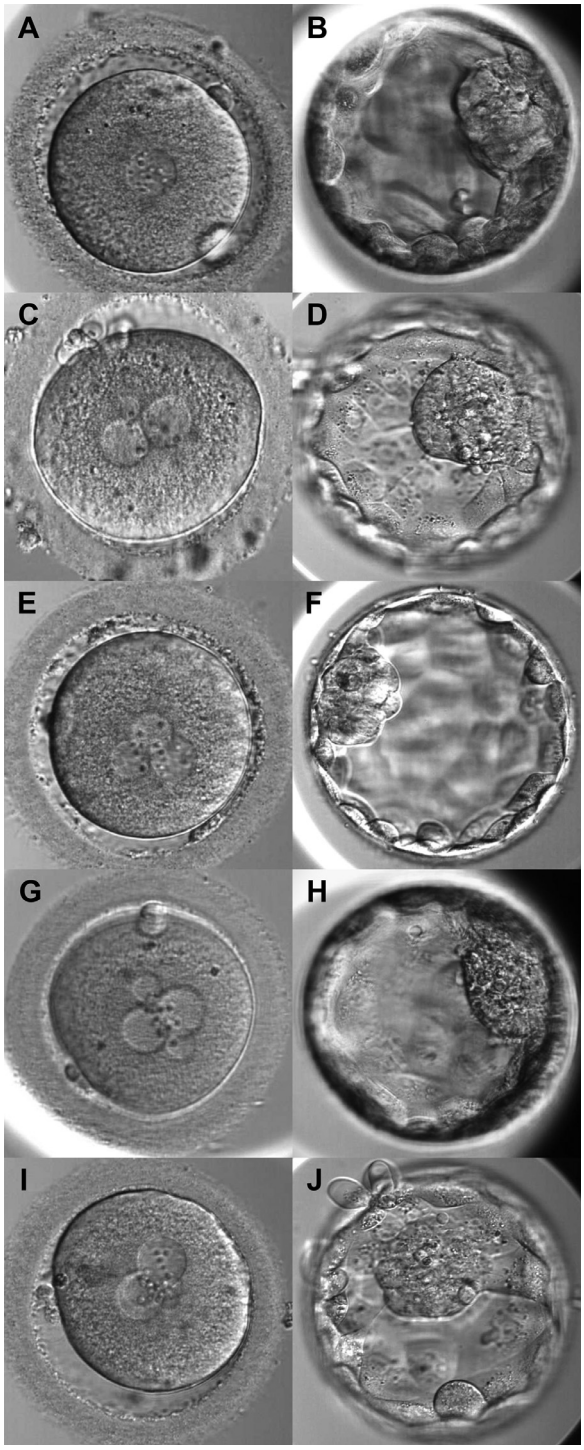


**FIGURE 1** Non-two pro-nuclei zygotes, and the resulting blastocysts after culture. (A, C, E, G, I) 1PN, 2.1PN, 3PN, 4PN and 5PN zygotes, respectively. The images to the right of each (B, D, F, H, J) are the corresponding resulting blastocysts.

Most of these embryos were derived from ICSI (89/152 [58.6%]), with the remainder generated via regular IVF (63/152 [41.4%]). The frequency of abnormal fertilization events did not differ between the two (Supplementary Table 3) (Fisher's exact test, $P = 0.51$), although there were proportionally more 2.1PN, 3PN and 4PN zygotes from ICSI and more 1PN and 5PN from IVF.

Examination of routine PGT-A cases also permitted the analysis of a larger cohort (4822) of 2PN embryos. Results show that

almost all (4737/4822 [98.2%]) the blastocysts derived from 2PN zygotes were diploid, with only a small fraction (85/4,822 [1.8%]) shown to be either polyploid (1.4%) or haploid (0.4%) (TABLE 1). Results also indicated that most (93/152 [61.2%]) of the 152 zygotes identified at the fertilization check stage as non-2PN were in fact diploid, and hence were normally fertilized (TABLE 1). Analysis of these data indicated that the proportions of haploid and polyploid embryos differed between zygote groups (Fisher's exact test with simulated *P*-values, $P = 1e\text{-}07$). Subsequent post-hoc comparison of the embryos resulting from abnormally fertilized zygotes to those resulting from 2PN zygotes indicated that embryos resulting from 1PN zygotes were more likely to be haploid, embryos resulting from 3PN zygotes were more likely to be polyploid as were embryos resulting from 4PN zygotes (all $P < 0.001$ in Bonferroni-corrected pairwise Fisher's exact tests). Other pairwise comparisons were non-significant (all $P \geq 0.09$ in comparisons by Fisher's exact test with embryos derived from 2PN zygotes), an expected result given the limited numbers of embryos derived from 0PN, 2.1PN, 4PN and 5PN zygotes.

Interestingly, 44 out of the 89 (49.4%) embryos derived from ICSI were either haploid or polyploid, a significantly higher proportion than that seen in the embryos derived from IVF, of which 16 of the 63 (25.4%) embryos were haploid or polyploid (Fisher's exact test, $P = 0.004$). As parental cheek swabs had been obtained from the parents of these embryos, the parental origin of the haploidy and polyploidy could be assessed (Supplementary Table 4). Analysis of these data indicated that a maternal origin of polyploidy was much more likely in zygotes derived from ICSI than from IVF (Fisher's exact test, $P = 0.005$).

Preimplantation genetic testing for aneuploidy analysis also indicated that many (49.1%) of the 93 diploid embryos derived from abnormally fertilized zygotes were also euploid (TABLE 2). Analysis of these data indicated the proportions of euploid and aneuploid embryos did not differ between zygote groups (Fisher's exact test with simulated *P* values, $P = 0.10$). That is, these data indicate that diploid embryos from abnormally fertilized zygotes that develop to the blastocyst stage are as likely to be euploid as those from 2PN zygotes. Further to this, three of these euploid embryos did not extrude a second polar body.

Our PGT-A analysis also allowed the characterization of aneuploidy type (TABLE 2). Analysis of the distribution of abnormality type, stratified as simple (two or fewer chromosomes affected) or complex (more than two chromosomes affected), indicated that the zygote groups did not differ in the patterns of aneuploidies that were detected (Fisher's exact test with simulated *P* values, $P = 0.12$).

At the time of writing, after obtaining informed consent, 12 of the euploid 1PN, 2.1PN and 3PN embryos had been transferred. The outcomes of these transfers can be seen in TABLE 3, with these data not further analysed given the limited number of transfers.

## DISCUSSION

The present study focused on the analysis of embryos that were assessed as abnormal at the fertilization check, but that, when left in culture, completed development to the blastocyst stage. Preimplantation genetic testing for aneuploidy of these embryos indicated that that most were diploid and, therefore, that normal fertilization had occurred. Over 60% of the embryos identified as abnormal at the fertilization check were in fact normally fertilized. Of these, over 40% were euploid and suitable for transfer. These results are broadly in line with other studies that have identified euploid embryos among those identified as abnormally fertilized (*Capalbo et al., 2017*; *Lim and Lee, 2019*; *Mutia et al., 2019*; *Canon et al., 2023*; *Mizuta et al., 2023*). Given that three of the embryos identified as euploid in this study did not extrude a second polar body, both key elements of the fertilization check are called into question.

Several potential explanations are available for the observed results. The pseudo-extrusion of chromosomes when the second polar body is extruded, for example, may be a potential explanation for the occurrence of diploid embryos with more than two pronuclei. Retention of chromosomes from the second polar body, even though the polar body itself is expelled, could result from a defect in the oocyte machinery, leading to a haploid set of chromosomes that should have been ejected remaining within the oocyte. In these cases, although syngamy may proceed normally, leading to the formation of a diploid zygote with a complete set of

**TABLE 1 PLOIDY STATUS OF 152 EMBRYOS CLASSIFIED AS 0PN, 1PN, 2.1PN, 3PN, 4PN AND 5PN AT THE FERTILIZATION CHECK, TESTED BY PREIMPLANTATION GENETIC TESTING FOR ANEUPLOIDY**

| Pronuclear status | PGT-A tested embryos, *n* | Diploid embryos, *n* (%) | Haploid Embryos, *n* (%) | Polyploid embryos, *n* (%) |
|---|---|---|---|---|
| 0PN | 2 | 1 (50) | 0 (0) | 1 (50) |
| 1PN | 50 | 38 (76) | 10 (20)[a] | 2 (4) |
| 2.1PN | 24 | 24 (100) | 0 (0) | 0 (0) |
| 3PN | 65 | 25 (38.5) | 7 (10.8) | 33 (50.8)[a] |
| 4PN | 9 | 3 (33) | 0 (0) | 6 (67)[a] |
| 5PN | 2 | 2 (100) | 0 (0) | 0 (0) |
| Total non-2PN | 152 | 93 (61.2) | 17 (11.2) | 42 (27.6) |
| 2PN | 4822 | 4,737 (98.2) | 17 (0.4) | 68 (1.4) |

Embryos identified as diploid, haploid or polyploid with the number (and percentage) shown. For comparison, the 4822 embryos that were classified as 2PN were also assessed for ploidy status (established from routine screening) using preimplantation genetic testing for aneuploidy (PGT-A).

[a] Proportions differ from those in the 2PN zygotes; $P < 0.001$ by post-hoc Bonferroni-corrected pairwise Fisher's exact tests, with other comparisons to the 2PN embryos all $P > 0.09$.

**TABLE 2 PREIMPLANTATION GENETIC TESTING FOR ANEUPLOIDY RESULTS FOR DIPLOID EMBRYOS**

| Pronuclear status | Total diploid embryos, *n* | Euploid, *n* (%) | Total aneuploid (including mosaics), *n* (%) | Aneuploid embryos with two or fewer chromosomes involved, fraction (%) | Aneuploid embryos with more than two chromosomes involved, fraction (%) |
|---|---|---|---|---|---|
| 0PN | 1 | 0 (0) | 1 (100) | 1/1 (100) | 0/1 (0) |
| 1PN | 38 | 21 (55.3) | 17 (44.7) | 12/17 (70.6) | 5/17 (29.4) |
| 2.1PN | 24 | 8 (33.3) | 16 (66.7) | 10/16 (62.5) | 6/16 (37.5) |
| 3PN | 25 | 9 (36) | 16 (64) | 7/16 (43.8) | 9/16 (56.3) |
| 4PN | 3 | 0 (0) | 3 (100) | 2/3 (66.7) | 1/3 (33.3) |
| 5PN | 2 | 1 (50) | 1 (50) | 0/1 (0) | 1/1 (100) |
| Total non-2PN | 93 | 39 (41.9) | 54 (58.1) | 32/54 (59.3) | 22/54 (40.7) |
| 2PN | 4,737 | 1,639 (34.6) | 3098 (65.4) | 2238/3098 (72.2) | 860/3098 (27.8) |

Preimplantation genetic testing for aneuploidy (PGT-A) results for diploid embryos indicating the number (and percentage) that were euploid and aneuploid (including mosaics), and a comparison of the nature of the aneuploidies. Analysis of the proportions of euploid and aneuploid embryos between zygote groups tested using Fisher's exact test with simulated *P* values; $P = 0.10$.

chromosomes, the additional set of chromosomes from the retained second polar body may not participate in syngamy but instead persist as an extra pronucleus. Over time, these additional chromosomes degrade or fail to contribute to embryo development. Further to this, abnormally fertilized embryos may also result from the retention of the second polar body, which is usually extruded during the final stages of meiosis. This would result in the zygote containing both its own pronucleus and the additional pronucleus from the retained polar body, potentially resulting in polyploidy.

Importantly, the overall rate of euploidy or aneuploidy seen here was not significantly different between embryos derived from abnormally fertilized zygotes and those derived from 2PN zygotes (TABLE 2). Abnormally fertilized zygotes that develop to the blastocyst stage and are diploid, therefore, had the same chance of being euploid as those developing from 2PN zygotes. These data therefore indicate that abnormally fertilized zygotes may be a valuable source of transferable embryos, particularly in cases in which 2PN zygotes are absent or limited. The small sample size of 0PN, 4PN, and 5PN embryos is, however, a limiting factor in this study and, as such, generalized conclusions about these categories cannot be made. At the time of writing, the transfer of euploid embryos derived from 1PN, 2.1PN and 3PN zygotes have all resulted in healthy live births, with additional pregnancies ongoing. This further supports the view that healthy babies can result from the transfer of abnormally fertilized embryos, providing they are chromosomally normal. Many similar studies have focused on one type of abnormal fertilization; for example, *Kobayashi et al. (2021)* and *Lui et al. (2016)* focused on 0PN embryos, whereas *Li et al. (2020)* concentrated on 1PN embryos. In contrast, despite the relatively small dataset presented here, we showed that all types of abnormal fertilization events can result in euploid embryos. As a result, we tentatively propose an order in which abnormally fertilized embryos should be considered for PGT-A testing and potential subsequent transfer. Specifically, we would advocate for the prioritization of 1PN embryos, followed by 2.1PN and then 3PN embryos, based on their diploid rates (TABLE 1).

Interestingly, PGT-A testing of different types of abnormally fertilized embryos showed that a large amount of variation within the different abnormality types. For example, some non-euploid 3PN embryos were triploid, whereas others were polyploid. Comparatively simple aneuploidies were also seen in the dataset; for example, although many of the non-euploid 1PN embryos were haploid, most were found to be aneuploid with abnormalities in two or fewer

**TABLE 3 OUTCOMES OF NON-2PN TRANSFERRED EMBRYOS**

| Outcomes | 1PN (maternal age 37.7 ± 5.8) | 2.1 PN (maternal age 39 ± 3.5) | 3PN (maternal age 37.5 ± 0.7) |
|---|---|---|---|
| Embryos transferred | 6 | 3 | 3 |
| Negative beta-HCG test | 4 (66.7) | 0 (0) | 1 (33.3) |
| Biochemical pregnancy/miscarriage[a] | 0 (0) | 1 (33.3) | 0 (0) |
| Ongoing pregnancy[b] | 0 (0) | 1 (33.3) | 1 (33.3) |
| Live birth | 2 (33.3) | 1 (33.3) | 1 (33.3) |

Maternal age (years) is presented as mean ± standard deviation. Data presented as number (%).

[a] A loss between positive pregnancy test and 6 weeks of gestation.

[b] Ongoing pregnancy at time of publication, after fetal heartbeat detected.

chromosomes. This study also found that the most of the ICSI polyploidy was digynically derived, perhaps indicating that the oocyte chromosomal spindle could be negatively affected by this type of ART technology. The use of the latest sequencing technology also highlights that diploid embryos only having maternal or paternal chromosomes could be detected and eliminated as these could lead to pregnancy loss through ovarian teratomas or hydatidiform moles. It would be unwise, therefore, to transfer embryos derived from non-2PN zygotes without appropriate genetic testing. This suggests a potential unexpected advantage of PGT-A. Specifically, critics of PGT-A argue that it can reduce the number of embryos available for transfer and hence the cumulative live birth rates (*Griffin and Oğur, 2018*; *Victor et al., 2020*). These data however demonstrate that if PGT-A is used to screen embryos derived from abnormally fertilized zygotes, it is possible to make additional embryos available for transfer. If we assume that around 30% of embryos are non-2PN (*Papale et al., 2012*; *ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017*) and about 40% of these are euploid (results from this study) then, for the first time, PGT-A has the potential to improve cumulative pregnancy rates, formerly something it was considered unable to do. With mean reported numbers of blastocysts being 2.44 for IVF and 2.11 for ICSI (*Speyer et al., 2019*), then any significant increase would lead to more successful assisted reproduction for thousands of people.

In alignment with others (*Capalbo et al., 2017*; *Doody, 2021*), we, therefore, contend that a re-evaluation of the fertilization check step in ART is warranted. The significant advances being made in the development and use of prediction algorithms in ART (*Fukunaga et al., 2020*; *Chapple et al., 2023*) and in the field of artificial intelligence, may further assist in optimal embryo assessment and selection (*Shen et al., 2022*).

In conclusion, the fertilization check, when embryologists examine embryos for signs of successful fertilization between 16- and 18-h after oocyte and sperm co-incubation, or after ICSI, is still a part of routine clinical practice in ART laboratories. It has been for over 40 years. Given the developments that have been made in the field throughout this time, a growing body of evidence suggests that 'abnormal fertilization' events may be inappropriately named. It may now be the time, therefore, to re-evaluate this standard practice across the sector, particularly when PGT-A is indicated.

## DATA AVAILABILITY

Data will be made available on request.

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## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2024.104595.

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