Effect of three pro-nuclei (3PN) on the outcomes of PGT

XIAO-HUA WU (xiaohuawu65@126.com)
The Fourth Hospital of Shijiazhuang, Shijiazhuang Obstetrics and Gynecology Hospital affiliated to Hebei Medical University

yan jiang
The Fourth Hospital of Shijiazhuang, Shijiazhuang Obstetrics and Gynecology Hospital affiliated to Hebei Medical University

JING-CHUAN YUAN
The Fourth Hospital of Shijiazhuang, Shijiazhuang Obstetrics and Gynecology Hospital affiliated to Hebei Medical University

GE SONG
The Fourth Hospital of Shijiazhuang, Shijiazhuang Obstetrics and Gynecology Hospital affiliated to Hebei Medical University

CAI-PING GENG
The Fourth Hospital of Shijiazhuang, Shijiazhuang Obstetrics and Gynecology Hospital affiliated to Hebei Medical University

XU-HUI ZHANG
The Fourth Hospital of Shijiazhuang, Shijiazhuang Obstetrics and Gynecology Hospital affiliated to Hebei Medical University

Research Article

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Abstract

Aims

To explore the influence of 3PN on outcomes in preimplantation genetic testing (PGT) cycles.

Methods

This was a retrospective study of patients undergoing PGT treatment included 139 PGT for aneuploidy (PGT-A) cycles and 285 cycles PGT for monogenic/single gene defects and/or chromosomal structural rearrangements (PGT-M/SR). The 424 PGT cycles were divided into two groups as follows: group 1 included 343 cycles with no 3PN zygotes and group 2 included 81 cycles with 3PN zygotes. 3PN cycle rate was compared between PGT-A and PGT-M/SR cycles. The outcomes of PGT were analyzed between the two groups.

Results

The female and male's age were significantly higher in PGT-A than PGT-M/SR cycles. Whereas 3PN rate was not significantly different between the two groups. The number of retrieved oocytes was significantly higher in 3PN group than Non 3PN group (13.98 ± 7.10 vs. 10.89 ± 6.65; P < 0.05). The MII oocyte rate, 2PN fertilization rate, D3 high embryo rate, biopsy blastocyst rate, per oocyte utilization and D5 blastocyst rate were significantly lower in 3PN group than Non 3PN group (P < 0.05). The results of chromosomal mosaic rate was significantly higher in 3PN group than Non 3PN group (16.3 vs. 11.9; P < 0.05).

Conclusions

No correlation was observed between 3PN formation rate and PGT-A or PGT-M/SR cycles. The occurrence of 3PN seems to impair the developing blastocyst and interfere with good embryo formation rate and mosaic rate in PGT. But the occurrence of 3PN does not seem to impair the euploid rate and aneuploid rate.

Introduction

Three pro-nuclei (3PN) are one of the most common abnormalities deviated from the expected bipronuclear pattern (2PN). Many investigators have evaluated the clinical and laboratory factors associated with the occurrence of 3PN[1, 2]. The presence of 3PN in both conventional IVF (cIVF) and ICSI cycles was associated with the higher E₂ and greater number of retrieved oocytes [2].

Some researchers maintained that the 3PN rate may serve as a surrogate marker of oocyte quality that represents the integrity of the oocytes in the entire recruited cohort and a prognostic indicator for IVF
cycle outcome using embryos derived from normally fertilized oocytes[1, 3]. The clinical pregnancy rate of elective single blastocyst stage embryo transfer (eSBET) in 3PN group was significantly lower than no 3PN group[3]. The higher proportions of 3PN affected embryos led to worse clinical outcomes in ICSI cycles[4, 5, 6].

However, seldom study researched the effect of 3PN on the outcomes of preimplantation genetic testing (PGT) and the influence of patients with 3PN on the trophectoderm biopsy results of aneuploidy and chromosomal abnormalities by performed PGT. PGT included PGT for aneuploidy (PGT-A) and PGT for monogenic/single gene defects and/or PGT for chromosomal structural rearrangements (PGT-M/SR).

The present study aimed to analyze 3PN rate in PGT-A and PGT-M/SR. PGT need both ICSI and eSBET. So it's unclear if 3PN might make a negative effect on outcomes in PGT. And biopsy results between 3PN fertilization occurred cycles and not recurred cycles.

**PATIENTS AND METHODS**

**Patients**

This retrospective study included 424 PGT cycles in which 139 PGT-A cycles and 285 PGT-M/SR cycles between February 2020 and January 2023. Eighty-one cycles with 3PN zygotes and 343 cycles without 3PN zygotes were observed in 424 PGT cycles. Written patient consent was obtained after providing a thorough explanation. This study was conducted with the approval of the ethics committee of the Fourth Hospital of Shijiazhuang (approval number 20220139)

**Stimulation, Oocyte Retrieval, Fertilization, Embryo culture and Scoring**

Most patients used the standard long protocol and antagonist protocol for controlled ovarian hyperstimulation (COH). COH and oocyte retrieval has been previously described by Yan Jiang, et al.[7]. Sperm used for routine insemination procedure using a standard method. Oocytes used for ICSI were treated with bovine hyaluronidase (Sigma-Aldrich, St Louis, MO, USA) for dispersing the cumulus cells after 39 ~ 40 h of trigger. ICSI was performed to all MII oocytes. The embryos were placed into the G-1 culture system (VitroLife, Sweden) medium and covered with mineral oil. Fertilization was confirmed by the presence of two pronuclei 16–18 h after ICSI (Day 1).

“High quality embryos” should have 7–9 cells on day 3, contain less than 20% fragments, but might be a little uneven in appearance. On day 3 all available embryos transferred into G-2 culture medium in group culture (Vitrolife, Sweden). In the morning of D5 or D6, blastocysts were scored by two experienced embryologist using the system of Gardner and Schoolcraft[8]. Blastocysts with a score ≥ 3, including those with grades BC, CB were selected on day 5 and day 6 for blastomere biopsy and vitrification.

**Blastomere biopsy and vitrification**
Blastomere biopsy was performed using a laser beam (Hamilton Thorne, Beverly, MA, USA). The blastomere was aspirated from the embryo and released into the medium for amplification (YK001B, Yikon, China). The amplification results were detected by copy number variation (CNV) analysis and next generation sequencing (Illumina, San Diego, CA, USA).

The procedure was always performed using one blastocyst for each straw. The blastocyst was moved at room temperature (22–25°C) to Kitazato (Japan) equilibration solution (ES). After 8 min, the blastocyst was quickly washed in vitrification solution (VS) for 45–60s and transferred onto the straw (Kitazato Japan) using a micropipette and immersed vertically into liquid nitrogen[7].

Data Analysis

Statistical analyses were performed using SPSS 19.0 statistical software (SPSS Inc.). The results are presented as the mean ± standard deviation (SD). The mean values of two groups were compared using the independent samples t-test. Percentages were compared using the χ2 test and P< 0.05 was considered statistically significant.

Results

1. Basic condition in PGT-A and PGT-M/SR cycles

In 424 PGT cycles, there were 19.1% (81/424) cycles observed 3PN fertilization. Number of 3PN zygotes per cycle was 1.31 (106/81) and 3PN zygote rate was only 11.4% (106/929) (Table 1).
Table 1
Basic condition in PGT-A and PGT-M/SR cycles

<table>
<thead>
<tr>
<th></th>
<th>PGT</th>
<th>PGT-A</th>
<th>PGT-M/SR</th>
<th>χ² / t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>cycle (n)</td>
<td>424</td>
<td>139</td>
<td>285</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>COH-anti rate</td>
<td>67.9</td>
<td>79.1</td>
<td>62.5</td>
<td>11.932</td>
<td>0.001*</td>
</tr>
<tr>
<td>(288/424)</td>
<td>(110/139)</td>
<td>(178/285)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient age (years)</td>
<td>33.17 ± 5.45</td>
<td>37.69 ± 4.55</td>
<td>30.97 ± 4.40</td>
<td>14.609</td>
<td>0.000</td>
</tr>
<tr>
<td>Male age (years)</td>
<td>33.94 ± 6.23</td>
<td>38.65 ± 6.20</td>
<td>31.64 ± 4.79</td>
<td>11.722</td>
<td>0.000</td>
</tr>
<tr>
<td>Severe male factor</td>
<td>6.3</td>
<td>4.3</td>
<td>7.4</td>
<td>1.460</td>
<td>0.227</td>
</tr>
<tr>
<td>(27/424)</td>
<td>(6/139)</td>
<td>(21/285)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oocyte number</td>
<td>11.48 ± 6.84</td>
<td>8.24 ± 5.32</td>
<td>13.05 ± 6.95</td>
<td>7.874</td>
<td>0.000</td>
</tr>
<tr>
<td>3PN cycle rate</td>
<td>19.1</td>
<td>15.8</td>
<td>20.7</td>
<td>1.436</td>
<td>0.231</td>
</tr>
<tr>
<td>(81/424)</td>
<td>(22/139)</td>
<td>(59/285)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3PN rate</td>
<td>11.4</td>
<td>15.2</td>
<td>10.5</td>
<td>3.290</td>
<td>0.070</td>
</tr>
<tr>
<td>(106/929)</td>
<td>(28/184)</td>
<td>(78/745)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3PN per cycle</td>
<td>1.31</td>
<td>1.27</td>
<td>1.32</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>(106/81)</td>
<td>(28/22)</td>
<td>(78/59)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

There were 15.8% (22/139) cycles observed 3PN fertilization in PGT-A cycles. And 20.7% (59/285) in PGT-M/SR cycles. 3PN cycle rate and 3PN rate were not significantly different between the PGT-A and PGT-M/SR cycles (15.8% vs.20.7%; 15.2% vs.10.5%, \( P > 0.05 \)) (Table 1).

The female and male's age, COH-anti rate were significantly higher in PGT-A than PGT-M/SR cycles. The oocyte number was significantly lower in PGT-A than PGT-M/SR cycles. Severe male factor was not significantly different between the two groups (Table 1).

2. Outcomes between 3PN group and Non 3PN group in PGT cycles

There was no significant difference in COH protocol, female's age, BMI, male age, the degradation, and 2PN cleavage rates between 3PN group and Non 3PN group (\( P > 0.05 \)). The number of retrieved oocytes was significantly higher in 3PN group than Non 3PN group (13.98 ± 7.10 vs. 10.89 ± 6.65; \( P < 0.05 \)). However, number of blastocyst per cycle was no significantly difference between two groups (3.92 ± 3.55 vs.3.30 ± 2.70, \( P > 0.05 \)). The MII oocyte rate, 2PN fertilization rate, D3 high embryo rate, blastocyst rate, per oocyte utilization and D5 blastocyst rate were significantly lower in 3PN group than Non 3PN group (\( P < 0.05 \)) (Table 2).
Table 2  
Outcomes between 3PN group and Non 3PN group in PGT cycles

<table>
<thead>
<tr>
<th></th>
<th>Non 3PN group</th>
<th>3PN group</th>
<th>$\chi^2/t$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGT cycle (n)</td>
<td>343</td>
<td>81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COH-anti rate</td>
<td>67.6 (232/343)</td>
<td>69.1 (56/81)</td>
<td>0.067</td>
<td>0.795</td>
</tr>
<tr>
<td>COH-Long rate</td>
<td>24.2 (83/343)</td>
<td>22.2 (18/81)</td>
<td>0.141</td>
<td>0.707</td>
</tr>
<tr>
<td>Other COH rate</td>
<td>8.2 (28/343)</td>
<td>8.6 (7/81)</td>
<td>0.020</td>
<td>0.888</td>
</tr>
<tr>
<td>BMI</td>
<td>23.03 ± 3.46</td>
<td>22.89 ± 3.78</td>
<td>0.311</td>
<td>0.691</td>
</tr>
<tr>
<td>Patient age (years)</td>
<td>33.22 ± 5.46</td>
<td>32.98 ± 5.44</td>
<td>0.366</td>
<td>0.715</td>
</tr>
<tr>
<td>Male age (years)</td>
<td>33.86 ± 6.24</td>
<td>34.28 ± 6.25</td>
<td>0.546</td>
<td>0.585</td>
</tr>
<tr>
<td>Severe male factor</td>
<td>6.4 (22/343)</td>
<td>6.2 (5/81)</td>
<td>0.015</td>
<td>0.910</td>
</tr>
<tr>
<td>Oocyte number</td>
<td>10.89 ± 6.65</td>
<td>13.98 ± 7.10</td>
<td>3.711</td>
<td>0.000*</td>
</tr>
<tr>
<td>MII oocyte rate</td>
<td>86.9 (3244/3734)</td>
<td>82.1 (929/1132)</td>
<td>16.457</td>
<td>0.000*</td>
</tr>
<tr>
<td>Degradation rate</td>
<td>3.4 (109/3244)</td>
<td>4.1 (38/929)</td>
<td>1.134</td>
<td>0.287</td>
</tr>
<tr>
<td>2PN fertilizaion rate</td>
<td>90.4 (2933/3244)</td>
<td>78.3 (727/929)</td>
<td>98.99</td>
<td>0.000*</td>
</tr>
<tr>
<td>2PN Cleavage rate</td>
<td>99.2 (2909/2933)</td>
<td>99.4 (723/727)</td>
<td>0.551</td>
<td>0.458</td>
</tr>
<tr>
<td>D3 high embryo rate</td>
<td>50.0 (1455/2909)</td>
<td>42.6 (308/723)</td>
<td>12.753</td>
<td>0.000*</td>
</tr>
<tr>
<td>Blastocyst rate</td>
<td>46.2 (1343/2909)</td>
<td>36.9 (267/723)</td>
<td>20.023</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

(*$P < 0.05$)
<table>
<thead>
<tr>
<th></th>
<th>Non 3PN group</th>
<th>3PN group</th>
<th>$\chi^2/t$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>per oocyte utilization</td>
<td>36.0</td>
<td>23.6</td>
<td>60.137</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>(1343/3734)</td>
<td>(267/1132)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blastocyst per cycle</td>
<td>3.92 ± 3.55</td>
<td>3.30 ± 2.70</td>
<td>1.473</td>
<td>0.141</td>
</tr>
<tr>
<td>D5 blastocyst rate</td>
<td>67.2</td>
<td>60.0</td>
<td>5.197</td>
<td>0.023*</td>
</tr>
<tr>
<td></td>
<td>(902/1343)</td>
<td>(160/267)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Successful biopsy rate</td>
<td>98.4</td>
<td>98.5</td>
<td>0.006</td>
<td>0.937</td>
</tr>
<tr>
<td></td>
<td>(1322/1343)</td>
<td>(263/267)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>euploidy rate</td>
<td>39.7</td>
<td>36.1</td>
<td>1.188</td>
<td>0.276</td>
</tr>
<tr>
<td></td>
<td>(525/1322)</td>
<td>(95/263)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aneuploid rate</td>
<td>48.4</td>
<td>47.5</td>
<td>0.068</td>
<td>0.794</td>
</tr>
<tr>
<td></td>
<td>(640/1322)</td>
<td>(125/263)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mosaic rate</td>
<td>11.9</td>
<td>16.3</td>
<td>3.982</td>
<td>0.046*</td>
</tr>
<tr>
<td></td>
<td>(157/1322)</td>
<td>(43/263)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(*$P< 0.05$)

The results of chromosomal euploid and aneuploid rate were not significant difference. However, the mosaic rate was significantly higher in 3PN group than Non 3PN group (16.3 vs. 12.1; $P<0.05$) (Table 2).

**Discussion**

The incidence of 3PN fertilization was 2.5–6.2% in ICSI cycles[1]. The mechanisms resulting in polyspermy fertilization formation is still unclear. Different mechanisms were suggested between c-IVF and ICSI cycles[1]. Immunofluorescent analysis shows that most of 3PN zygotes derived from c-IVF had a female PN (fPN) and two male PNs (mPN), suggesting polyspermy[9]. On the other hand, 3PN zygotes after ICSI showed two fPNs and a mPN, indicating failure of the extrusion of the second polar body[4, 9, 10, 11]. In the 3PN embryos after ICSI, equal ratios of XXX and XXY were observed and no XYY embryos were present[12]. However, Time-lapse technology (TLT) has revealed extrusion of the second polar body was observed in a vast majority of 3PN zygotes (92.1%) in ICSI cycles[13].

3PN incidence may serve as a prognostic indicator for IVF cycle outcome using embryos derived from normally fertilized oocytes and make a negative effect on pregnancy outcomes in eSBET[3, 4, 14]. However, Ye YH et al showed that polyspermic fertilization is correlated with improved oocyte receptivity to sperm and could be considered as an encouraging sign for the success of cIVF[15]. While some studies showed that 3PN incidence might be caused by oocyte quality or sperm abnormality in cIVF.
cycles[16], and the high clVF 3PN incidence (3PN > 20%) may predict poor outcomes in blastocyst-stage embryo transfer cycles, but not influence for cleavage-stage pregnancy outcome[17].

The higher proportions of 3PN also affected embryos led to worse clinical outcomes in ICSI cycles[4]. When the 3PN rate was less than 20% or 25%, the ICSI outcomes were not impaired[5, 6]. Only when the 3PN zygote rate was > 20% or > 25%, the proportion of 3PN zygotes after ICSI serves as a negative prognostic indicator for ICSI cycle outcome[5, 6]. However, This study showed that the number of 3PN zygotes per cycle was only 1.31(106/81). The presence of 3PN can serve as a surrogate marker of PGT outcomes for the remaining cohort of zygotes. 3PN zygote rate > 20% or > 25% was fewer, so we did not analysis the influence of the difference 3PN zygote rate.

In couples with a normal semen analysis undergoing ICSI, the 3PN rate may serve as a surrogate marker of oocyte quality and the 3PN rate is a significant predictor of implantation rate for the remaining cohort of zygotes[4]. The starting and total dose of gonadotropins administered and the total days of stimulation are independent predictors of the 3PN rate[4]. High proportion of 3PN zygotes made a negative effect on clinical outcomes for IVF-ET cycles with long-term protocol[18]. 3PN formation may also be increased in women that are high responders to gonadotropins [4]. We also analysis the COH protocol, there were no significantly difference in COH-anti and COH-Long rate between the 3PN group and Non 3PN group.

However, more retrieved oocytes could result in 3PN incidence easily which is consistent with previous study [19]. The reason is that once more oocytes were retrieved, they display a more degenerated, immature, and aging phenotype[19]. In this study, we also observed more retrieved oocytes in 3PN group than Non 3PN group. However, number of blastocyst per cycle was no significantly difference between two groups (3.92 ± 3.55 vs.3.30 ± 2.70, P > 0.05). And lower MII oocyte rate, 2PN fertilizaion rate, D3 high embryo rate, blastocyst rate, per oocyte utilization and D5 blastocyst rate in 3PN group than Non 3PN group (P<0.05).

Both maternal and paternal ages were higher in cases involving 3PN fertilization in ICSI[13]. However, in this study although female and male's ages were significantly older in PGT-A cycles than PGT-M/SR cycles. 3PN cycle rate was no significantly different in two groups(15.8% vs. 20.7%, P > 0.05). And the patients age was no significant difference between 3PN group and Non 3PN group. Similar as Sachs et al. result that no significant association of aging between women who had 3PN zygotes after ICSI[20]. Our study first comparing the 3PN rate between PGT-A cycles and PGT-M/SR cycles(15.2% vs.10.5%, P > 0.05).

No correlation was observed between the semen analysis abnormalities and the 3PN formation rates in ICSI[21]. Some studies suggest severe sperm abnormalities and oocyte aging can contribute to the process[4, 22]. Male factor infertility impacts the rate of mosaic blastocysts in cycles of PGT-A[23]. In this study, we did not observed correlation between the semen analysis abnormalities and the 3PN formation rates in PGT cycles.
Some studies researched the relationship between Anti-Müllerian hormone (AMH), luteinizing hormone (LH) and 3PN. AMH significantly correlates with the presence of multiple pronuclei in the zygote. The presence of multiple pronuclei increased when AMH levels were higher\cite{24}. Supplementation of exogenous LH activity to ovarian stimulation may be associated with lower prevalence of 3PN zygotes in IVF cycles, but not in ICSI cycles\cite{1}. 3PN zygotes may occur less frequently in LH activity-added cycles\cite{1}.

Nowadays most reference laboratories require use of ICSI and blastocyst biopsy for PGT\cite{25}. The results presented here suggest that 3PN rate do not affect the euploidy and aneuploid rate of the blastocyst. However, the mosaic rate was significantly higher in 3PN group than Non 3PN group (16.3 vs. 12.1; \( P < 0.05 \)). There was a preliminary study revealed not all morphologically 3PN embryos are genetically abnormal\cite{26}. Some 3PN zygotes, with two normal-sized PNs and an additional smaller PN (2.1PN) embryos were diploid, however, predominantly aneuploid, and therefore could not be used for embryo transfer\cite{27}. The deficiency of this study was that lack of analyzing the 3PN blastocysts genetic testing.

In conclusion, No correlation was observed between 3PN formation rate and PGT-A or PGT-M/SR cycles. the presence of 3PN in PGT cycles was associated with the greater number of retrieved oocytes. The occurrence of 3PN seems to impair the developing blastocyst and interfere with good embryo formation rate and mosaic rate in PGT. But the occurrence of 3PN does not seem to impair the euploid rate and aneuploid rate.

**Declarations**

**Ethics approval and consent to participate**

The Fourth Hospital of Shijiazhuang Ethics Committee approved this study (approval no. 20220049). Informed consent was obtained from all subjects. The procedures used in this study adhered to the tenets of the Declaration of Helsinki. All experiments were performed in accordance with relevant guidelines and regulations.

**Consent for publication**

Not applicable

**Availability of data and materials**

The datasets used during the present study are available from the corresponding author upon reasonable request. Data will be made available to the editors of the journal for review or query upon request.

**Competing interests**

All authors declare no conflict of interest.

**Funding**
Authors' contributions

XHW designed the study and revised the paper. YJ and JCY wrote the main manuscript text. GS, CPG, and XHZ prepared table 1-2. All authors reviewed the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Not applicable

References


