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Original Article

Three pro-nuclei (3PN) incidence factors and clinical outcomes: a retrospective study from the fresh embryo transfer of in vitro fertilization with donor sperm (IVF-D)

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Abstract: *Objectives*: The aim of this study was to explore the main factors of 3PN incidence and determine whether the presence of 3PN could lead to a worse pregnancy outcome. *Methods*: This study included 508 IVF-D (in vitro fertilization with donor sperm) cycles from January 2013 to September 2014. The patients were divided into three groups as follows: group 1 included patients with no 3PN zygotes, group 2 included patients with 1%-25% 3PN zygotes and group 3 included patients with > 25% 3PN zygotes. *Results*: We observed that more retrieved oocytes and higher HCG day peak E2 value could result in 3PN incidence more easily. When the 3PN zygotes rate was > 25%, the percentages of normal fertilization (68.4% and 66.3% and 46.4%, $P < 0.001$), day 3 grade I+II embryos (41.2% and 38.6% and 25.8%, $P < 0.001$), day 3 grade I+II+III embryos (68.7% and 65.2% and 61.4%, $P = 0.032$) and implantation rates (52.1% and 50.8% and 45.4%, $P = 0.026$) were significantly lower than that in the other two groups respectively. The pregnancy rate was lower in 3PN > 25% group than that in the other two groups but there was no significant difference (65.2% and 66.7% and 55.6%, $P = 0.266$). The cleavage (98.3% and 97.2% and 98.2%, $P = 0.063$) and early abortion (7.1% and 8.0% and 8.6%, $P = 0.930$) rate were identical among three groups. *Conclusions*: More retrieved oocytes and higher HCG day peak E2 value could result in 3PN incidence more easily. Interestingly, normal fertilization rate, day-3 grade I+II embryos rate, day-3 grade I+II+III embryos rate and implantation rate were significantly lower in IVF-D cycles with a 3PN incidence of > 25%. The number of day-3 grade I+II embryos might be a key factor for pregnancy in IVF-D cycles with a 3PN incidence of > 25%.

Keywords: IVF-D, 3PN, pregnancy

Introduction

Three pro-nuclei (3PN) prevalence among all pregnancies has been estimated to be approximately 1% to 3% [1, 2], whereas it accounts for 15%-18% of cytogenetically abnormal cases among spontaneous abortions. The abortion usually occurs in the early development period but a few cases with unusually long survival have been reported. 3PN formation exist results from two circumstances: the combination of one maternal and two paternal sets or the combination of one paternal and two maternal sets [3, 4]. It is believed that 3PN results either from polyspermic fertilization or oocyte-derived meiotic failure. Polyspermy should not occur in intracytoplasmic sperm injection (ICSI) since only one sperm is injected into each oocyte. So 3PN incidence is due mostly to retention of the second polar body after ICSI [5, 6]. However, it is unknown whether the source of sperm might impair subsequent emission of the second polar body. The present 3PN studies on clinical outcomes were mainly from ICSI cycles to exclude polyspermy. IVF outcome depends highly on oocyte quality, so a high incidence of 3PN in ICSI cycles may suggest an occult oocyte property that may serve as an independent predictor of IVF cycle outcome [7-9]. During conventional IVF, however, the block to polyspermy may not always occur. Some studies have suggested that the 3PN incidence is a result of advanced maternal age or severe sperm abnormality in IVF cycles [10]. In other words, 3PN incidence may be associated with oocyte quality when the semen was normal. It is still unknown whether 3PN incidence may serves as a prognostic indicator for

IVF cycle outcome using embryos derived from normally fertilized oocytes with normal semen. In the current study, we selected the donor semen for study which was strictly checked by the laboratory technicians. The semen obtained from donor has strict standards in morphology, concentration, motility and chromosome analysis. We attempt to assess whether 3PN frequency correlates with clinical outcomes in IVF-D cycles.

Materials and methods

This study was a retrospective analysis of the data from our center and was approved by the Ethics Review Board of Northwest Women's and Children's Hospital.

This study contained 508 IVF-ET cycles with fresh, non donated oocytes and required the help of donor semen between January 2013 and September 2014. Every male patient had been diagnosed that the semen extracted by testicular sperm aspiration (TESA) could not meet the ICSI criteria before into the IVF-D cycles. Our demand for donor semen: semen volume > 2 ml, concentration > 60×10^6/ml, a+b grade sperm ≥ 50% and patients were healthy and had no chromosome abnormality. The entry criteria included all female patients were younger than 38 years old to eliminate possible age-related cycle characteristics and all first-attempt down-regulated ovarian stimulation cycles were standard long protocols.

Ovarian stimulation

All patients used the standard long protocols with GnRH agonist (GnRH-a, Decapeptyl Germany) and recombinant FSH (GONAL-f, Merck Serono Italy; Puregon, Organon Netherlands) for controlled ovarian hyperstimulation (COH). 10, 000 units of human chorionic gonadotrophin (hCG) were administered when > 3 follicles were > 18 mm. Oocyte retrieval was performed 36 h later by transvaginal ultrasonography-guided aspiration.

Origin of sperm and fertilization

The semen for IVF-D was freezing samples. The semen would be incubated for 10 min in 37°C water bath and observed under the high-power microscope. After confirming the samples meet the fertilization criteria, it could be performed. In conventional fertilization, each egg is incubated with approximately 40,000 sperm and the culture volume is 0.7 ml. Generally two eggs are co-cultured with sperms in each well. Evaluation and selection for embryo transfer

Embryonic cleavage and morphologic appearance were assessed 64 to 68 h after IVF. A morphologic score was given for day-3 embryo according to the number of blastomeres, homogeneous degree of blastomeres and degree of cytoplasmic fragmentation: grade I (8-10 blastomeres, even homogeneous blastomeres < 10% cytoplasmic fragmentation), grade II (6-7 or > 10 blastomeres with even homogeneous blastomeres of no cytoplasmic fragmentation; 8-10 blastomeres, even homogeneous blastomeres with 10%-20% cytoplasmic fragmentation), grade III (uneven and non-homogeneous blastomeres with 20-50% cytoplasmic fragmentation), and grade IV(uneven and non-homogeneous blastomeres with > 50% cytoplasmic fragmentation). Grade I and Grade II was identified as high-quality embryo [11].

ET and pregnancy confirmation

The ET catheter (COOK IRELAND LTD, Ireland) was used for transfers. Before transfer, any vaginal and cervical secretions were gently removed from the vagina/cervix with small pledgets of cotton wool, moistened with warm normal saline. The mucus in the cervical canal was wiped away. After transfer, the catheter was checked for retained embryo sand the presence of blood. After ET, all patients were given luteal support (Duphaston; progesterone injection). Clinical pregnancy was confirmed by the presence of a gestational sac [11].

Statistical analysis

Data were analyzed using the SPSS 17.0 for Windows. Statistical significance for parametric data was assessed by using the Student's t-test, and the Mann-Whitney U test was used for nonparametric data. Differences between proportions were computed by using the χ^2 test or the Fisher exact test, as appropriate. Differences were considered statistically significant at $P < 0.05$. When $P < 0.05$ in three groups, data were compared between groups.

Results

For the entire study, 249 cycles were characterized by the presence of zygotes with 3PN,

Table 1. Characteristics of patients and stimulation cycles

Characteristics of the study groups	3PN = 0%	3PN > 0%	*P* Value
No. of patients (n)	259	249	/
Female's age (y)	29.2±4.1	28.7±3.9	0.482
Total gonadotrophin dose (ampule)	26.8±8.5	25.9±8.8	0.148
Mean length of stimulation (d)	10.1±1.8	10.1±1.6	0.912
Basal Serum FSH (mIU/ml)	6.2±1.5	6.3±1.6	0.437
Basal Serum E2 (pg/ml)	41.4±32.6	42.3±31.28	0.622
No. of retrieved oocytes (n)	10.9±5.5	13.6±6.1	< 0.001
HCG day peak E2 (pg/mL)	4516±2368	5684±2500	< 0.001

Table 2. Comparison of the characteristics of patients

Characteristics of the study groups	3PN = 0% (N = 259)	3PN = 1-25% (N = 186)	3PN > 25% (N = 63)	*P* Value
Female's age (y)	29.2±4.1	28.2±3.8	29.4±4.2	0.554
Endometrial thickness (mm)	12.3±2.4	12.4±2.3	11.7±1.8	0.638
Infertile time (yr)	4.1±3.1	4.1±3.3	3.7±2.9	0.362
Oocytes retrieved (n)	10.9±5.5[a]	15.2±6.9[a,b]	11.0±5.2[b]	< 0.001
Embryos transferred (n)	1.8±0.4	1.7±0.5	1.8±0.4	0.823

Data were compared between groups. *P*-values: a vs. a < 0.001; b vs. b < 0.001.

whereas no incidence of 3PN zygotes was observed in 259 cycles. The distribution of cycle-specific parameters between groups is described in **Table 1**, and no significant difference were observed in female's age, total gonadotrophin dose, basal serum FSH value, basal serum E2 value or mean length of stimulation between the two groups. However, patients in 3PN > 0% group had higher peak serum E2 levels and more oocytes retrieved than 3PN = 0% group (P < 0.001) (**Table 1**).

To compare the clinical outcomes in different 3PN rate, the cycles were split into three groups according to 3PN zygotes rate: no 3PN zygotes detected (3PN = 0%, N = 259), 3PN = 1-25% (N = 186) and 3PN > 25% (N = 63). No significant difference was observed among three groups in female's age, endometrial thickness, infertile time or number of embryos transferred. The number of retrieved oocytes were higher in Group 3PN = 1-25% vs. Group 3PN = 0% (15.2% and 10.9%, *P* < 0.001) and Group 3PN = 1-25% vs. Group 3PN > 25% (15.2% and 11.0%, *P* < 0.001), but there was no significant difference in Group 3PN = 0% vs. Group 3PN > 25% (10.9% and 11.0%, *P* = 0.776) (**Table 2**). The cleavage (98.3% and 97.2% and 98.2%, *P* = 0.063), pregnancy (65.2% and 66.7% and 55.6%, *P* = 0.266) and early abortion (7.1% and 8.0% and 8.6%, *P* = 0.930) rate were no significant difference among three groups. When the 3PN zygote rate was > 25%, normal fertilization (68.4% and 66.3% and 46.4%, *P* < 0.001), day-3 grade I+II embryos (41.2% and 38.6% and 25.8%, *P* < 0.001), day-3 grade I+II+III embryos (68.7% and 65.2% and 61.4%, *P* = 0.028) and implantation (52.1% and 50.8% and 45.4%, *P* = 0.034) rate were significantly lower than that in the other two groups. However, between 3PN = 0% group and 3PN = 1-25% group, normal fertilization (68.4% and 66.3%, P = 0.224), day-3 grade I+II embryos (41.2% and 38.6%, P = 0.083), day-3 grade I+II+III embryos (68.7% and 65.2%, *P* = 0.268) and implantation rate (52.1% and 50.8%, *P* = 0.472) showed no significant difference (**Table 3**).

We compared the characteristics of 3PN > 25% patients in pregnant group and not pregnant group, there was no significant difference in female's age, endometrial thickness, infertile time, number of oocytes retrieved or number of embryos transferred (*P* > 0.05). The cycle proportion of no good-quality embryo in not pregnant group was slightly higher than that in pregnant group, but there was no significant difference (*P* = 0.328) (**Table 4**).

Discussion

3PN results either from polyspermic fertilization or from oocyte-derived meiotic failure [12, 13]. One feature shared by all eggs is that they must fuse with one-and only one-sperm [14, 15]. The reasons for causing 3PN fertilization are complicated. Some investigators have suggested that the incidence of 3PN fertilization after IVF is a result of advanced maternal age or severe sperm abnormalities and other investigators have suggested that the propensity toward 3PN is a function of ovarian stimulation, as indicated by high peak E2 levels, large

Table 3. Clinical outcomes according to 3PN incidence

Parameter	3PN = 0% (N = 259)	3PN = 1-25% (N = 186)	3PN > 25% (N = 63)	*P* Value
Cleavage rate (%)	98.3	97.2	98.4	0.061
Normal fertilization rate (%)	68.4[a]	66.3[b]	46.4[a,b]	< 0.001
Day-3 grade I+II embryos rate(%)	41.2[a]	38.6[b]	25.8[a,b]	< 0.001
Day-3 grade I+II+III embryos rate (%)	68.7[c]	65.2[d]	61.4[c,d]	0.028
Implantation rate (%)	52.1[c]	50.8[d]	43.4[c,d]	0.034
Pregnancy rate (n, %)	169 (65.2)	124 (66.7)	35 (55.6)	0.266
Early abortion rate (n, %)	12 (7.1)	10 (8.0)	3 (8.6)	0.930

Data were compared between groups. *P*-values: a vs. a < 0.005; b vs b < 0.001; c vs. c < 0.05; d vs. d < 0.05.

Table 4. Comparison of. the characteristics of 3PN > 25% patients

Parameter	Pregnant Group (3PN > 25%, N = 38)	Not Pregnant Group (3PN > 25%, N = 25)	*P* Value
Female's age (y)	29.6±3.6	29.2±3.2	0.442[a]
Endometrial thickness (cm)	12.1±2.0	11.4±1.8	0.517[a]
Infertile time (yr)	3.9±2.9	4.2±3.6	0.361[a]
Oocytes retrieved (n)	11.3±4.2	11.8±4.6	0.274[a]
Embryos transferred (n)	1.8±0.4	1.8±0.4	0.863[a]
No. of good-quality embryos (cycle, %)			0.328[b]
N = 0	4 (10.5)	8 (32.0)	
N = 1-2	26 (68.4)	12 (48.0)	
N ≥ 3	8 (21.1)	5 (20)	

[a]t-test; [b]Mann-Whitney U-test.

oocyte yields, high gonadotropin doses, and lengthy stimulations [16]. As for the association of high sperm concentrations with polyspermy in IVF Wolf et al. [17] clearly demonstrated that an increase in sperm concentration is directly related to an increase in the incidence of polyspermy. Plachot et al. [18] showed that a lower incidence of polyspermy when sperm quality was reduced. Levitan et al. [19] showed that egg size has influence on the risk of polyspermy.

In this retrospective study, 508 fresh IVF-ET cycles with donor sperm were involved. The 3PN incidence cycle rate was 49.02% and 3PN zygotes rate was 10.02%. The patients were divided into two groups as follows: 3PN > 0% group and 3PN = 0% group. We observed no significant difference in female's age, total gonadotrophin dose, basal serum FSH, basal serum E2 or mean length of stimulation between the two groups. However, our results revealed that patients in 3PN > 0% group had higher peak serum E2 levels and more oocytes retrieved which was consistent with most investigators' study. The probable cause of no significant difference in total gonadotropin dose and mean length of stimulation were not clear which implied unrecognized confounding factors that were not measured.

There is limited investigation describing whether variables in the ovarian stimulation cycle can be used as predictors for evaluating 3PN formation. Most recently a case-control study was performed to identify factors associated with the formation of 3PN zygotes. 3PN formation resulting from standard IVF insemination has been investigated, and the majority of evidence suggests that in most instances it is due to polyspermy [20]. Although there were a lot of factors that could result in 3PN fertilization, the fertilization process is extremely precise, and there is no doubt that multiple layers of safety mechanisms exist to ensure the achievement of normal fertilization. Two types of mechanisms for polyspermy block have been reported: the "oocyte membrane block" and the "zona reaction" which were relatively important for normal fertilization [21, 22]. Mio et al. [23]

succeeded in demonstrating the possible existence of a novel mechanism of polyspermy block in human oocytes by use of original time-lapse cinematography system. Bianchi et al. [15] observed a new protein name Juno, which could prevent polyspermy in fertilization and is highly expressed on unfertilized eggs. Juno became undetectable within 30-40 min after fertilization, in close agreement with the timing of the membrane block to polyspermy. The mechanisms for polyspermy block was complicated and effective to ensure normal fertilization occurrence, so 3PN incidence might in a large part be caused by oocyte quality or sperm abnormality [24]. In this study, we selected semen of donors to exclude abnormal sperm influence as much as possible in IVF-ET cycles. When 3PN zygotes detected, it might be caused by dysfunctional oocyte. It was no doubt that polyspermy should not occur in ICSI because only one sperm is injected into each oocyte, and if 3PN incidence, it must be the abnormalities of oocyte function. However, it might not provide more accurate information for predicting oocyte and embryo competence in IVF cycles because the fertilization mechanism was different between IVF and ICSI.

3PN formation resulting from standard IVF fertilization could be due to polyspermy. There was no doubt that 3PN formation gave rise to the decreased number of day-3 grade I+II+III embryos. Because of conflicting reports, it has been controversial whether 3PN formation correlates with clinical outcomes. If it could not cause a worse clinical outcome, the prevention of 3PN formation seems to be unnecessary. Dayal et al. [25] observed that pregnancy, implantation, clinical-pregnancy, and live-birth rates were significantly higher in the cohort of patients who had < 20% of embryos appearing 3PN, compared with the group who had > 20% of zygotes appearing triploid. Rosen et al. [6] showed that 3PN formation in this subset of patients is a significant negative predictor of implantation. Figueira et al. [13] observed an approximately 50% lower risk of pregnancy and a 3.5-fold higher risk of miscarriage in cycles with a 3PN incidence of > 25%. The study mentioned above was from ICSI cycles to minimize diandric causes of 3PN and provide us some prediction information for conventional IVF cycles. Shibahara et al. [7] maintained that prevention of multinucleate formation seems to be unnecessary because a higher successful IVF-ET outcome is expected as a result of the excellent fertilization rate in these patients. Qin et al. [8] retrospectively analyzed their IVF-ET data and observed that Polyspermy rate in IVF-ET was no significant effects on the implantation rate and pregnancy rate. Zeng et al. [26] and Yang et al. [27] believed that higher 3PN rate could decrease pregnancy rate in conventional IVF-ET cycle but the number of cases was limited in their study. The distinction between our research and other study was that we selected high quality donor semen which was different from conventional IVF. It might exclude the impacts of abnormal sperm almostly and provide us more precise cues in predicting the clinical outcomes of 3PN incidence in the fertilization of IVF. It is well-known that the time of embryos transfer was relatively significant for pregnancy and implantation outcomes. In our study, the fresh embryos for transfer contain D3 embryos and D5 blastocyst. The D5 blastocyst proportion in 3PN = 0%, 3PN = 1-25% and 3PN > 25% group was 24.6%, 30.2% and 22.8 respectively, however, there was no significant difference among three groups ($P > 0.05$). So it might not an influence factor contributing to the difference of pregnancy and implantation outcomes in the three groups.

Our results demonstrated an inverse correlation between 3PN incidence and IVF-D outcomes. Cycles with a high proportion of 3PN zygotes displayed lower percentages of normal fertilization, day-3 grade I+II embryos, day-3 grade I+II+III embryos and implantation rate. Nevertheless, we did not observe any significant difference in the abortion rate among the three groups. The pregnancy rate in 3PN > 25% group was lowest but there was no significant difference. The cause we analyzed was that limited cases of 3PN > 25%. Another possibility is that in most of 3PN > 25% cycles we selected two embryos for transfer but the probability of obtained two sacs was lower compared with other two groups. In the characteristics comparison of 3PN > 25% patients by clinical pregnancy, the patients and cycle characteristics were no significant difference between pregnant group and not pregnant group. The probability of no good-quality embryos was higher compared with not pregnant group which indicated that embryos quality was an important factor related to pregnancy outcome.

In conclusion, our findings suggest that prediction of 3PN formation in human IVF-D cycles might be possible in patients when the number of oocytes collected is higher and HCG day peak E2 value is higher. In addition, prevention of high proportion zygotes appearing 3PN seems to be necessary because it would decrease normal fertilization, day-3 grade I+II embryos, day-3 grade I+II+III embryos and implantation rate. There is no doubt that a prospective study is needed to further confirm our finding.

Disclosure of conflict of interest

None.

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