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Blastocyst Formation and Aneuploidy Rate of Tripronuclear Embryos

Tripronükleer Embriyolarda Gözlenen Blastulasyon ve Anöploidi Oranı

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ABSTRACT

Objective: Human embryos generated from abnormal fertilization are usually discarded in assisted reproductive technologies (ART) practice. This study aims to investigate the potential of developing into blastocysts and the chromosomal constitution of tripronuclear (3PN) zygotes. **Material and Methods:** This study consists of patients who had at least one 3PN embryo after a conventional ART cycle and a subsequent preimplantation genetic testing for aneuploidy (PGT-A) between 2020 and 2022 in our IVF unit. Demographic data, cycle outcomes, blastulation and euploidy rates of 3PN zygotes were retrospectively analyzed. **Results:** Among 108 patients with a history of PGT-A for diverse indications, 17 had a total of 29 embryos with abnormal pronuclei (3PN). Of those, 9 embryos achieved to develop into a blastocyst and, 6 of them were furtherly evaluated with a fluorescence in situ hybridization (FISH) analysis. The ratio of 3PN embryos to all fertilized embryos (3PN/2PN+3PN) was %21. Although 31% of 3PN embryos reached to the blastocyst stage, all of the biopsied embryos were reported to be aneuploid and clinically discarded later. **Conclusion:** Based on the low/null euploidy rate; it is mandatory to perform PGT-A before the transfer of a 3PN-derived embryo, particularly in poor prognosis patients with a limited number of embryos. Instead of genetic analysis, routine discard of 3PN-derived blastocysts would be more cost-effective in women with normal ovarian response. Further randomized prospective studies are required to determine the subgroup of patients who could benefit more from genetic testing of 3PN zygotes.

Keywords: Tripronuclear; blastocyst; euploidy

ÖZET

Amaç: Klinik uygulamada, YÜT (yardımcı üreme teknikleri) sonrası anormal fertilizasyon gözlenen embriyoların transferi genellikle tercih edilmemektedir. Bu çalışmada, tripronükleer zigotların (3PN) blastosist aşamasına ulaşma oranları ve kromozomal yapılarının incelenmesi amaçlanmıştır. **Gereç ve Yöntemler:** Kliniğimizde 2020-2022 yılları arasında preimplantasyon genetik test-anöploidi taraması (PGT-A) uygulanan hastalardan 3PN yapısında embriyoya sahip olanlar bu retrospektif çalışmaya dahil edildi. Demografik data, siklus sonuçları, 3PN zigotların blastulasyon ve öploidi oranları kayıt altına alındı. **Bulgular:** Çeşitli nedenlerle PGT-A uygulanmış olan 108 hastanın kayıtları incelendiğinde, 17 hastaya ait toplam 29 embriyonun 3PN yapısında olduğu tespit edildi. Blastosist aşamasına ulaşan 9 embriyodan 6'sına fluorescence in situ hybridization (FISH) testi uygulandı. 3PN embriyoların, tüm embriyolara oranı (3PN/2PN+3PN) %21 olarak hesaplandı. 3PN embriyoların %31'i blastosist aşamasına ulaşmasına rağmen, biyopsi yapılan tüm embriyolarda anöploidi tespit edilmesi üzerine imha edilmeleri planlandı. **Sonuç:** Düşük öploidi oranı nedeniyle, 3PN embriyoların transferi kötü prognozlu ve kısıtlı sayıda embriyo elde edilebilmiş hasta grubunda PGT-A testi sonrası düşünülebilir. Normal over yanıtı olan hastalarda gelişen 3PN kökenli blastosistlerin genetik test yerine imhaya bırakılması daha maliyet-etkin bir yaklaşım olabilir. 3PN embriyoların genetik analizinden daha çok fayda görmesi beklenen hasta subgrubunun belirlenmesi açısından ileri prospektif çalışmalar gereklidir.

Anahtar Kelimeler: Tripronükleer; blastosist; öploidi

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Zygote formation is the first step of embryo development, occurring after the union of the spermatozoon and oocyte. The existence of two even pronuclei clarifies a successful fertilization.[1] However, this complex process may result in abnormally fertilized tripronuclear (3PN) zygotes at a rate of 2.5%-6.2% after intracytoplasmic sperm injection (ICSI) and 5.0%-8.1% after in vitro fertilization (IVF).[2-4] The parental origin also differs between these two methods. While the majority of 3PN-IVF zygotes are of dispermic origin, the 3PN-ICSI zygotes are digynic.[5]

Assessment of embryonic development according to the kinetic and morphologic features provides information to select embryos with high implantation potential in assisted reproduction treatment (ART). While the blastocyst transfer results in a higher implantation rate, reaching to the blastocyst stage can not directly contribute to euploidy even in normally fertilized embryos (2PN).[6] Consistent with this, neither parental inheritance nor ploidy determines the progression of 3PN embryos to the blastocyst stage.[7]

3PN-derived embryos may be responsible for 15-18% of chromosomal abnormalities among spontaneous abortions and several fetal abnormalities.[8] Therefore, human embryos generated from abnormal fertilization are usually discarded in clinical practice. This study aims to investigate the potential of developing into blastocysts and the chromosomal constitution of tripronuclear (3PN) zygotes.

MATERIAL AND METHODS

This study was conducted in compliance with the Declaration of Helsinki for Medical Research and approved by the ethics committee of Bahcesehir University (2023-07/01). The patients who had at least one 3PN embryo after a conventional ART cycle and a subsequent PGT-A between 2020 and 2022 in our IVF unit were recruited in the study. Informed consent was obtained from all participants for future use.

The patients were treated with flexible GnRH antagonist protocol (Cetrotide 0.25 mcg, Merck-Serono). Daily injection of individualized doses of recombinant follicle-stimulating hormone (rec-FSH) (Gonal-F, Merck-Serono) and/or human menopausal gonadotropin (Menogon, Ferring) was started on day 3 of the cycle and continued until at least three dominant follicles reached a diameter of 17 mm. Chorigonadotropin alfa (Ovitrelle 250 mcg (6500 IU), Merck-Serono) or/and triptorelin acetate (Gonapeptyl, Ferring) was administered for final maturation, followed by transvaginal oocyte retrieval 34-36 h later. Prior to intracytoplasmic sperm injection (ICSI), cumulus-oocyte-complexes (COCs) were denuded using hyaluronidase. The first assessment of the pronuclei was performed using an inverted microscope 16-18 hours following ICSI. The percentage of fragmentation, blastomere count and size were evaluated at the early cleavage stages and then cultured until day 5 or 6. The embryos that reached the blastocyst stage were assessed using Gardner's score based on the degree of expansion, intracellular mass (ICM) and trophectoderm (TE). High-quality embryos were biopsied on D3/D5 or D6 by laser to be analyzed for chromosomal status using the fluorescence *in situ* hybridization (FISH) method as described before.[5]

STATISTICAL ANALYSIS

The Statistical Package for Social Sciences 20 (SPSS, SPSS Inc, Chicago) was used for data analysis. The distribution of the data was analyzed with Kolmogorov–Smirnov and Shapiro–Wilks tests. Descriptive analyses were given using tables of frequencies and percentages for the categorical variables and using mean and standard deviation or median and range for continuous variables.

RESULTS

Among 108 patients with a history of PGT-A for diverse indications (maternal age, recurrent implantation failure and recurrent pregnancy loss), 17 patients with a mean age of 35.23±6.81 had at least one 3PN embryo after a conventional ART. Cycle characteristics of the patients are presented in Table 1.

Briefly; out of 29 embryos with 3PN, 9 achieved to develop into a blastocyst. Of those, 6 embryos were furtherly evaluated with a fluorescence *in situ* hybridization (FISH) analysis. The ratio of 3PN embryos to all fertilized embryos (3PN/2PN+3PN) was %21. The blastocyst formation rate of 3PN em-

TABLE 1: Results of characteristics, ICSI parameters, blastulation and euploidy rates of patients with 3PN embryos

Patient (n:17)	Mean±St.Dev
Female age (years)	35.23±6.81
Male age (years)	36.53±7.23
Oocytes retrieved	12.76±7.38
Mature oocytes	9.82±6.06
2PN	6.41±4.19
3PN	1.58±1.06
	Percentage (%)
3PN/2PN+3PN	21
Blastulation rate	31
Euploidy rate	0

bryos was 31% (Table 1). While all of the biopsied embryos were reported as aneuploid (4 triploid, 1 complex polyploid and 1 complex aneuploid), no embryo transfer could be performed (Table 2).

DISCUSSION

The retention of the second polar body (2PB) mainly due to meiotic division failure of oocytes results in digynic triploidy. 3PN embryos that occurred after abnormal fertilization may present with normal early cellular cleavage, but development to blastocyst-stage may be arrested or aneuploidy may occur later.[9] It has been postulated that neither parental inheritance nor ploidy determines the progression of 3PN embryos to the blastocyst stage.[8] Similarly, we observed a blastulation rate of %31 but a null rate of euploidy in the recent study.

In a previous study, the blastulation rate of 2PN and 3PN embryos was reported as 60.8% and 42.8%, respectively.[10] Grau et al., suggested that 62.5% of good prognosis ICSI-3PN embryos progressed to the blastocyst stage.[8] In a study involving clinically discarded embryos, 3PN embryos revealed a blastocyte formation rate of 11.2% and after SNP array-based chromosomal analysis, 47.4 % of them were found in normal chromosomal constitution.[11] Mutia et al. reported an aneuploidy rate of 66.7% with the highest percentage of triploidy among 3PN embryos using Next Generation Sequencing (NGS).[12] Feenan and Herbert demonstrated that 61.8% of 3PN embryos had a triploid chromosome component, 25.2% had mosaic sequencing, and only 12.6% carried a diploid chromosome set.[13]

In clinical practice, 3PN embryos are usually discarded regarding the reported increased risk of miscarriages, neonatal deaths and fetal abnormalities including major central nervous system defects, abdominal wall defects and intrauterine growth retardation in spontaneous triploid pregnancies.[14,15] Thus, the transfer of a 3PN-derived embryo should be considered after the confirmation of normal chromosomal status by preimplantation genetic testing for aneuploidy (PGT-A). Consistent with this, a live birth after the transfer of a euploid tripronuclear embryo in a 36 years aged woman with diminished ovarian reserve has been reported.[16]

The capability of self-correction of the 3PN embryos or the microsurgical removal of the retained pronucleus may provide a chance to rescue 3PN embryos.[7,13] The limitation of the latter procedure involves the difficulty of choosing the right pronucleus to remove which could lead to inheriting two sets of maternal or paternal chromosomes. In a study by Haixia Jin et al., it was observed that the total blastulation

TABLE 2: Blastulation and chromosomal distribution of 3PN embryos.

	Age	Biopsy indication	Biopsy day	Blast/grade	FISH result
Patient 1	46	MA[a]	D5	3BB	Triploidy
Patient 2	41	MA[a]	D6	3CB	Complex polyploidy
Patient 3	36	RIF[b]	D3	3BB	Triploidy
			D3	Arrest	Triploidy
Patient 4	38	RPL[c]	D3	Early blast	Complex aneuploidy
Patient 5	39	MA[a]	D6	4BB	Triploidy

[a]MA: Maternal age; [b]RIF: Recurrent implantation failure; [c]RPL: Recurrent pregnancy loss

rate was significantly higher in the enucleated group (3→2PN zygotes) when compared to the 3PN group.[17] Kattara and Chen reported a healthy live birth with the karyotype 46XY after the removal of the pronucleus and the extra centrosome from a 3PN zygote.[18]

The limitations of this study were the retrospective design, the small sample size and the heterogeneity of the biopsy day. Notably; using the FISH method for genetic analysis of just 9 chromosomes was not a confounding factor in this study, because all biopsies detected an aneuploidy.

CONCLUSION

The existing evidence suggests that the majority of the embryos arising from zygotes with 3PN have abnormal chromosomal constitutions. Thus; it is mandatory to perform PGT-A before the transfer of a 3PN-derived embryo, particularly in cases with a limited number of embryos. Instead of genetic testing, routine discard of 3PN embryos would be more cost-effective in women with normal ovarian response. Further randomized prospective studies are required to determine the certain circumstances for genetic testing of 3PN zygotes.

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Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: *Timur Gürgan;* ***Design:*** *Timur Gürgan, Pınar Gülşen;* ***Control/Supervision:*** *Timur Gürgan;* ***Data Collection and/or Processing:*** *Pınar Gülşen;* ***Analysis and/or Interpretation:*** *Timur Gürgan, Pınar Gülşen;* ***Literature Review:*** *Pınar Gülşen;* ***Writing the Article:*** *Pınar Gülşen;* ***Critical Review:*** *Timur Gürgan.*

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