Effect of blastocyst morphology on the incidence of monozygotic twinning pregnancy after single blastocyst transfer: a retrospective cohort study

Xiaofei Ge
First Affiliated Hospital of Zhengzhou University
Jiaxin Zhang
First Affiliated Hospital of Zhengzhou University
Hao Shi
First Affiliated Hospital of Zhengzhou University
Zhiqin Bu (fccbuzq@zzu.edu.cn)
First Affiliated Hospital of Zhengzhou University
Yingpu Sun
First Affiliated Hospital of Zhengzhou University

Research Article

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Abstract

Background: Visual morphology-based selection for transfer is a currently common method with evaluation of blastocyst quality and prediction of pregnancy and live birth. The aim of this study is to explore whether blastocyst morphology [blastocyst stage, inner cell mass (ICM) and trophectoderm (TE) grading] has an impact on the occurrence of monozygotic twinning (MZT) after single blastocyst transfer (SBT).

Methods: All patients who obtained a clinical pregnancy after single blastocyst transfer between January 2015 and September 2021 were retrospectively included. Blastocyst morphology scores were assessed using Gardner grading system. Monozygotic twinning was defined as more than 1 gestational sac (GS), or 2 or more fetal heartbeats (FHB) in a single GS via ultrasound at 5-6 gestational weeks. MZT incidence associated with blastocyst morphological parameters was analyzed by multivariable logistic regression modeling.

Results: The overall MZT rate was 2.46% (227 of 9229 cases), of which was the highest in blastocysts of grade A TE and lowest in those with grade C TE (grade A: B: C= 3.40%: 2.67%: 1.58%, *P*=0.002). The higher risk of MZT pregnancy was associated with higher trophectoderm grading [grade A vs C: aOR, 1.883, 95% CI 1.069-3.315, *P*=0.028; grade B vs C: aOR, 1.559, 95% CI 1.066-2.279, *P*=0.022], but not with extended culture in *vitro* (day 5 vs day 6), vitrification (fresh vs frozen-thawed ET), assisted hatching (AH), blastocyst stage (stage 1-6) or ICM grading (A vs B).

Conclusions: TE grade is an independent risk factor of MZT after single blastocyst transfer. The effects of extended culture, AH, frozen-thawed procedures, blastocyst stage and ICM grade were not demonstrated.

Introduction

Multiple pregnancy is considered as a suboptimal outcome associated with infertility treatment [1], with the increase of adverse maternal and perinatal outcomes, such as preterm birth, low and very birthweight, stillbirth, and perinatal mortality [2], and mainly resulting from the manipulation of multiple embryo transfer. Monozygotic twinning (MZT) is caused by single zygote splitting, with a much higher risk than singleton and dizygotic pregnancies, such as twin-twin transfusion syndrome, twin anemia-polycythemia, and selective intrauterine growth restriction particularly in monochorionic twins. MZT accounts for 1.36-2.69% births after in *vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), which was much higher than that of 0.40-0.45% in natural conception births [3]. Over the past two decades, reproductive physicians have been working to reduce multiple pregnancies associated with infertility treatments [4] [5]. Multiple gestation rates have significantly reduced since elective single embryo transfer (eSET) was proposed and advocated in many IVF centers worldwide [6-10]. Nonetheless, MZT caused by early embryo splitting cannot be prevented by this strategy [11-13], which have aroused considerable clinical attention. Prior studies hypothesized that the increased MZT rate after IVF/ICSI might be related to IVF

laboratory procedures themselves [14] [15-18], including in *vitro* extended culture [19], embryo cryopreservation [13] and zona pellucida manipulations [20] [21], such as ICSI, assisted hatching (AH), embryo biopsy for preimplantation genetic testing (PGT). Although results among these heterogenous reports were inconsistent and the mechanisms of embryo splitting are still unclear, extended embryo culture (blastocyst culture) was identified a robust risk factor of embryo splitting [22, 23] .

Otsuki et al. [24] conducted an observation by time-lapse photography and proposed a hypothesis that the splitting susceptibility of inner cell mass (ICM) could be associated with arrangement features of ICM cells. They suggested that ICM with loosely grouped cells might be more susceptive to splitting than tightly packed counterparts. This hypothesis begun to link the inherent morphological characteristics of blastocyst to embryo splitting. The related reports conducted [25, 26] showed differences in the role of ICM and TE arrangement features in the occurrence of MZT after IVF/ICSI. Therefore, a retrospective cohort study including numerous SBT cycles was conducted to explore the association of inherent blastocyst morphological characteristics (blastocyst stage, grade ICM and TE) scored by Gardner grading system with the incidence of MZT after IVF/ICSI. This investigation may provide a novel blastocyst transfer guidance for limiting the risk of MZT after SBT.

Materials And Methods

Study design and population

All clinical pregnancies (n=9229) after SBT during January 2015 to September 2021 in the Reproductive Medical Center of The First Affiliated Hospital of Zhengzhou University were retrospectively included in this study. Only the first embryo transfer (ET) cycles with single blastocyst (PGT cycles were excluded) were included. Anonymous data were extracted from Clinical Reproductive Medicine Management System/Electronic Medical Record Cohort Database (CCRM/EMRCD) of the center. Ethical approval of this retrospective study was obtained by the institutional review board of our hospital.

Demographic characteristics of patients and parameters of blastocysts were extracted from our CCRM/EMRCD, including maternal age, body mass index (BMI), basal follicular stimulation hormone (FSH), type of cycles (fresh or frozen-thawed ET), ovarian stimulation procedures and insemination type (conventional IVF or ICSI, only in fresh cycles), assisted hatching (AH: yes or no), extended culture in *vitro* (day 5 or day 6), blastocyst stage (stage 1-6), ICM grading (A or B), TE grading (A, B or C), endometrium preparation protocols [natural cycle (NC) or hormone replacement therapy (HRT), only of frozen-thawed cycles], the Gardner score of blastocyst, and number of GS and FHB via ultrasound during the first trimester.

Laboratory protocols and blastocyst morphology parameters

The specific details of IVF/ICSI protocol and manipulation including insemination, embryo culture in *vitro*, vitrification and warming, endometrium preparation protocols of frozen-thawed ET have been described as published from our center [27]. Laser-AH was performed in frozen-thawed ET, previous implantation

failure and women older than 35 years of fresh cycles. Blastocyst morphological parameters including blastocyst stage, ICM grade and TE grade were elevated using the Gardner scoring system [28]. Blastocyst stage was classified from stage 1 to 6 according to blastocoel volume, blastocyst expansion degree and hatching status. Grade of ICM and TE was scored as “A, B or C” considering the cell number and the degree of junction. With grade C as a reference, ICM and TE grading A and B were regarded as higher grade. Only blastocyst with ICM grade of A or B were selected for transfer in our center.

Definition of outcome measures

Biochemical pregnancy was identified by the increasing level of serum β-hCG 14 and 18 days after ET. Clinical intrauterine pregnancies were confirmed when at least one intrauterine GS were seen via ultrasound scan 5-6 weeks after SBT, with or without FHBs. MZT pregnancy was identified according to the number of GS and FHB seen via ultrasound. Those with the presence of 2 or more GSs, or more than one FHB in single GS were defined as MZT, and another ultrasound was performed at 7-8 weeks for confirmation, while the remaining were singleton groups.

Statistical analysis

All statistics analyses were performed using SPSS Statistics (IBM, version 26.0). Mean ± standard deviation (SD) was used to express continuous data, and differences were compared by the student’s *t*-test. Categorical variables were presented as numbers with percentage and compared using Chi-squared test or the Fisher exact test. Multivariate logistic regression model was constructed to analyze the association between variables and the occurrence of MZT after SBT. *P* values (two-tailed) < 0.05 were considered statistically significant.

Results

Demographics and blastocyst characteristics of study subjects

Out of 9229 clinical pregnancies after SBT included in this study, 227 were defined as MZT pregnancy, including 149 pregnancies with monochorionic twins, and 5 pregnancies with monochorionic triplets 72 pregnancies with 2 GSs, and 1 pregnancy with 3 GSs. The brief flow chart was shown in Fig. 1. The overall MZT rate was 2.46%, while the remaining 9002 pregnancies with 1 GS and less than 2 FHBs were singleton as controls. Demographic and embryo characteristics were compared in Table 1. The incidence of MZT was significantly higher in vitro culture time of day 5 (day 5 vs day 6: 2.60% vs 1.65%; *P*=0.040), grade A of ICM (grade A vs B: 2.97% vs. 2.24%; *P*=0.038) and grade A of TE (grades A: B: C=3.40%: 2.67%: 1.58%; *P*=0.002). Other parameters, including maternal age, BMI, basal FSH, ovarian stimulation protocol (only fresh-ET cycles), endometrium preparation protocol (only frozen-thawed cycles), AH, the types of ET and blastocyst expansion stage, showed no significant difference between MZT and singleton groups.

Blastocyst morphological parameters and monozygotic twinning

Multivariate logistics regression analysis model between blastocyst morphological characteristics and the occurrence of MZT after SBT was showed in Table 2. In this model, there was no significant association between in *vitro* extended culture time (day 5 vs day 6), type of ET (fresh vs frozen-thawed ET), AH (yes vs no), blastocyst expansion stage, ICM grading (A vs B) and MZT. Alternatively, only TE grading was a significant independent factor related to the risk of MZT after SBT. Higher TE grading was associated with higher risk of MZT (A vs C: aOR 1.883, 95% CI 1.069-3.315, *P*=0.028; B vs C: aOR 1.559, 95% CI 1.066-2.279, *P*=0.022) (Fig. 2). Grade A TE exhibited the highest occurrence of MZT (3.40%), while the lowest was in grade C TE (1.58%) (Fig. 3).

Discussion

In this study, the overall MZT rate was 2.46% of 9229 clinical pregnancies after single fresh and frozen-thawed blastocyst transfer in our center, which was generally in line with previous reports [23]. The primary finding was the association between the risk of MZT and TE grade. Blastocysts with higher grade TE are more susceptible to resulting in MZT pregnancies. However, we failed to find the relation between MZT and embryo cryopreservation (fresh vs frozen-thawed ET), AH, and extended culture (day 5 vs day 6), compared to prior reports [15-17, 20]. Differences of these results might be caused by heterogeneity among these IVF centers, including the proportion of fresh and frozen-thawed cycles, blastocyst quality, and IVF-related laboratory manipulations, such as indications of AH and culture media.

Franasiak et al. [29]found that the incidence of MZT did not increase after transfer at blastocyst stage compared with cleavage stage ET when controlling the quality of embryo cohort, suggesting embryo characteristics might contribute to MZT rates. Prior studies on blastocyst morphology were mainly conducted to evaluate the quality and their predictive value for clinical outcomes. Otsuki et al. [24] observed 71 transferred frozen-thawed blastocysts by time-lapse observations and originally noted the hypothesis that compared with a blastocyst with tightly packed ICM cells, loosely grouped one was more conductive to splitting. Since then, the inner link of inherent blastocyst features and embryo splitting began to be discussed.

In the study examining the effects of hyaluronan-enriched transfer medium on MZT pregnancies after single frozen embryo transfer [30], they reported no association between embryo expansion, TE, ICM grade and the risk of MZT, which contradicted ours. All MZT embryos underwent PGT and part of them were cultured in a hyaluronan-enriched transfer medium, whereas PGT cycles were excluded in our cohorts. Nevertheless, TE grade was indicated a significant independent factor affecting the occurrence of MZT (A vs C: aOR 5.46, 95%CI 1.48-20.16, *P*=0.011; B vs C: aOR 3.96, 95%CI 1.17-13.40, *P*=0.027), but not the arrangement of ICM cells in the study including 2863 pregnancies after frozen-thawed SBT [26]. Combining the differences in serum hCG after ET between the MZT and singleton, they hypothesized that higher grade TE which was thought to be more well-developed might be associated with monozygotic splitting, potentially by increasing secretion of hCG. Our results agreed with their findings.

Meanwhile, in the study including 26254 clinical pregnancies after SBT from 4 centers conducted by Shi et al. [25], 402 pregnancies with sex concordance at birth were identified as MZT. Blastocysts with grade A ICM and grade B or C TE showed the lowest MZT rate, while counterparts with grade B or C ICM and grade A TE presented the highest**.** Blastocysts with lower grade ICM (B or C) and higher-grade TE (A) were with higher risk of MZT (aOR 2.62, 95%CI 1.60-4.43). They proposed a new hypothesis based on Otsuki's theory [24], which was that when loosely packed ICM cells might be more susceptible to splitting, higher grade TE with more number tightly arranged cells would provide further support in the development of splitting ICM resulting in MZT pregnancies.

Influenced by prior studies about the predictive power of blastocyst morphology for clinical outcomes [31-34], blastocysts with ICM score “C” are not routinely selected to transfer in our center for the higher efficiency of fertility treatment. Thus, our cohort included SBT cycles only with A and B grade ICM. Only TE grade was found to be associated with MZT, but not ICM grade. It could be related to the differences in the definition of MZT pregnancy, laboratory techniques and embryo culture system among different IVF centers. Our results were consistent with the conclusions about the role of TE grading in increasing MZT rate after IVF/ICSI of above reports considering blastocyst morphology. In addition, factors related to the susceptibility of ICM division were not found, and further scientific research is needed to explore the specific mechanism of ICM splitting.

The large sample size including 9229 clinical pregnancies after SBT from a single center is the main strength of this study. Although the assessment scores of blastocyst morphology based on Gardner scoring system were indeed influenced by subjectivity, our data was collected from a single center and evaluated by experienced embryologists followed by standard operating procedure, which has much reduced information bias. And there is a lack of more objective criteria for choice currently. Our study also has some limitations. First was the observational and retrospective nature. Second, data about ovarian stimulation procedures and fertilization methods of frozen-thawed cycles did not be recorded in our current CCRM/EMRCD. Therefore, the association between insemination methods and MZT occurrence was not analyzed in this study. Third, MZT pregnancy was mainly defined according to the presence of GSs and FHBs via ultrasound, but not confirmed with sex concordance at birth and the gold criteria of DNA profiling for zygosity, which might overlook the possibility of a concurrent natural conception, although this is a rare event.

Conclusion

In this study, we retrospectively investigated the association between inherent blastocyst morphological characteristics and risk of MZT occurrence after single blastocyst transfer. The results indicated that higher risk of MZT is associated with blastocysts with tightly packed trophectoderm cells, which may provide guidance of selection and order of blastocyst transfer to reduce the risk of MZT.

Abbreviations

MZT: Monozygotic twinning; SBT: Single blastocyst transfer; ICM: Inner cell mass; TE: Trophectoderm; ET: Embryo transfer; GS: Gestational sac; FHB: Fetal heartbeat; AH: Assisted hatching; IVF: In vitro fertilization; ICSI: Intracytoplasmic sperm injection; eSET: Elective single embryo transfer; PGT: Preimplantation genetic testing; BMI: Body mass index; FSH: Follicular stimulation hormone; GnRH: Gonadotropin-releasing-hormone; hCG: Human chorionic gonadotropin

Declarations

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Authors' contributions

YS, ZB, XG and JZ contributed to the study design, data analysis and manuscript preparation. HS handled patient recruitment and data collection. All authors read and approved the final manuscript.

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Availability of data and materials

All data supporting the conclusion of this article are included.

Ethics approval and consent to participate

The study has received approval and was carried out in accordance with the approved guidelines from the Zhengzhou University Research Ethics Board.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

References

1. **Multiple gestation pregnancy. The ESHRE Capri Workshop Group.** *Human reproduction (Oxford, England)*2000, **15:**1856-1864.
2. Hall JG: **Twinning.** *Lancet (London, England)*2003, **362:**735-743.

3. Murphy M, Hey K: **Twinning rates.** *Lancet (London, England)*1997, **349:**1398-1399.
4. **Multiple gestation associated with infertility therapy: an American Society for Reproductive Medicine Practice Committee opinion.** *Fertility and sterility*2012, **97:**825-834.
5. **Multiple gestation associated with infertility therapy: a committee opinion.** *Fertility and sterility*2022.
6. Farquhar C: **Avoiding multiple pregnancies in assisted reproductive technologies: transferring one embryo at a time should be the norm.** *Fertility and sterility*2020, **114:**671-672.
7. Chambers GM, Keller E, Choi S, Khalaf Y, Crawford S, Botha W, Ledger W: **Funding and public reporting strategies for reducing multiple pregnancy from fertility treatments.** *Fertility and sterility*2020, **114:**715-721.
8. Mancuso AC, Boulet SL, Duran E, Munch E, Kissin DM, Van Voorhis BJ: **Elective single embryo transfer in women less than age 38years reduces multiple birth rates, but not live birth rates, in United States fertility clinics.** *Fertility and sterility*2016, **106:**1107-1114.
9. Kanter JR, Boulet SL, Kawwass JF, Jamieson DJ, Kissin DM: **Trends and correlates of monozygotic twinning after single embryo transfer.** *Obstetrics and gynecology*2015, **125:**111-117.
10. **Elective single-embryo transfer.** *Fertility and sterility*2012, **97:**835-842.
11. Vega M, Zaghi S, Buyuk E, Jindal S: **Not all twins are monozygotic after elective single embryo transfer: analysis of 32,600 elective single embryo transfer cycles as reported to the Society for Assisted Reproductive Technology.** *Fertility and sterility*2018, **109:**118-122.
12. Noli L, Ogilvie C, Khalaf Y, Ilic D: **Potential of human twin embryos generated by embryo splitting in assisted reproduction and research.** *Human reproduction update*2017, **23:**156-165.
13. Osianlis T, Rombauts L, Gabbe M, Motteram C, Vollenhoven V: **Incidence and zygosity of twin births following transfers using a single fresh or frozen embryo.** *Human reproduction (Oxford, England)*2014, **29:**1438-1443.
14. Mateizel I, Santos-Ribeiro S, Done E, Van Landuyt L, Van de Velde H, Tournaye H, Verheyen G: **Do ARTs affect the incidence of monozygotic twinning?** *Human reproduction (Oxford, England)*2016, **31:**2435-2441.
15. Ikemoto Y, Kuroda K, Ochiai A, Yamashita S, Ikuma S, Nojiri S, Itakura A, Takeda S: **Prevalence and risk factors of zygotic splitting after 937 848 single embryo transfer cycles.** *Human reproduction (Oxford, England)*2018, **33:**1984-1991.
16. Liu X, Li P, Shi J: **Double trouble? Impact of frozen embryo transfer on the monozygotic twinning rate: a retrospective cohort study from 8459 cycles.** *Journal of assisted reproduction and genetics*2020, **37:**3051-3056.
17. MacKenna A, Schwarze JE, Crosby J, Zegers-Hochschild F: **Factors associated with embryo splitting and clinical outcome of monozygotic twins in pregnancies after IVF and ICSI.** *Human reproduction open*2020, **2020:**hoaa024.
18. Vaughan DA, Seidler EA, Murphy LA, Cleary EG, Penzias A, Norwitz ER, Sakkas D: **Double trouble? Clinic-specific risk factors for monozygotic twinning.** *Fertility and sterility*2020, **114:**587-594.

19. Kawachiya S, Bodri D, Shimada N, Kato K, Takehara Y, Kato O: **Blastocyst culture is associated with an elevated incidence of monozygotic twinning after single embryo transfer.** *Fertility and sterility*2011, **95:**2140-2142.
20. Liu H, Liu J, Chen S, Kang X, Du H, Li L: **Elevated incidence of monozygotic twinning is associated with extended embryo culture, but not with zona pellucida manipulation or freeze-thaw procedure.** *Fertility and sterility*2018, **109:**1044-1050.
21. Kamath MS, Antonisamy B, Sunkara SK: **Zygotic splitting following embryo biopsy: a cohort study of 207697 single-embryo transfers following IVF treatment.** *BJOG : an international journal of obstetrics and gynaecology*2020, **127:**562-569.
22. Hviid KVR, Malchau SS, Pinborg A, Nielsen HS: **Determinants of monozygotic twinning in ART: a systematic review and a meta-analysis.** *Human reproduction update*2018, **24:**468-483.
23. Busnelli A, Dallagiovanna C, Reschini M, Paffoni A, Fedele L, Somigliana E: **Risk factors for monozygotic twinning after invitro fertilization: a systematic review and meta-analysis.** *Fertility and sterility*2019, **111:**302-317.
24. Otsuki J, Iwasaki T, Katada Y, Sato H, Furuhashi K, Tsuji Y, Matsumoto Y, Shiotani M: **Grade and looseness of the inner cell mass may lead to the development of monochorionic diamniotic twins.** *Fertility and sterility*2016, **106:**640-644.
25. Shi W, Jin L, Liu J, Zhang C, Mi Y, Shi J, Wang H, Liang X: **Blastocyst morphology is associated with the incidence of monozygotic twinning in assisted reproductive technology.** *American journal of obstetrics and gynecology*2021, **225**.
26. Xu S, Zheng Q, Mo M, Xiong F, Hu X, Zeng Y: **High grade trophectoderm is associated with monozygotic twinning in frozen-thawed single blastocyst transfer.** *Archives of gynecology and obstetrics*2021, **304:**271-277.
27. Yang X, Bu Z, Hu L: **Live Birth Rate of Frozen-Thawed Single Blastocyst Transfer After 6 or 7 Days of Progesterone Administration in Hormone Replacement Therapy Cycles: A Propensity Score-Matched Cohort Study.** *Frontiers in endocrinology*2021, **12:**706427.
28. Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB: **Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer.** *Fertility and sterility*2000, **73:**1155-1158.
29. Franasiak JM, Dondik Y, Molinaro TA, Hong KH, Forman EJ, Werner MD, Upham KM, Scott RT: **Blastocyst transfer is not associated with increased rates of monozygotic twins when controlling for embryo cohort quality.** *Fertility and sterility*2015, **103**.
30. Kelly AG, Blakemore JK, McCaffrey C, Grifo JA: **Evaluation of clinical parameters as predictors of monozygotic twins after single frozen embryo transfer.** *F&S reports*2021, **2:**428-432.
31. Irani M, Reichman D, Robles A, Melnick A, Davis O, Zaninovic N, Xu K, Rosenwaks Z: **Morphologic grading of euploid blastocysts influences implantation and ongoing pregnancy rates.** *Fertility and sterility*2017, **107:**664-670.

32. Shi D, Xu J, Zhang M, Niu W, Shi H, Yao G, Li Y, Zhang N, Sun Y: **Association between the quality of inner cell mass and first trimester miscarriage after single blastocyst transfer.** *Reproductive biology and endocrinology : RB&E*2020, **18:**43.
33. Ai J, Jin L, Zheng Y, Yang P, Huang B, Dong X: **The Morphology of Inner Cell Mass Is the Strongest Predictor of Live Birth After a Frozen-Thawed Single Embryo Transfer.** *Frontiers in endocrinology*2021, **12:**621221.
34. Ross T: **P–200 To transfer or to discard: A retrospective analysis of ploidy, implantation and birthweight outcomes of grade "C" blastocysts following preimplantation genetic testing for aneuploidy (PGT-A).** *Human Reproduction*2021, **36**.

Tables

Table1 Demographic characteristics of patients and embryos in the MZT and singleton cohorts of single blastocyst transfer cycles

variable	Entire cohort	MZT (n, %)	Singleton	*P* value
No. of cycles	9229	227(2.46)	9002	
Maternal age (y)	30.26±4.22	29.99±3.67	30.26±4.23	0.272
<25	698	12(1.72)	686	0.100
25-29	3465	90(2.63)	3375	
30-34	3598	99(2.75)	3499	
35-39	1257	25(1.99)	1232	
≥40	211	1(0.47)	210	
BMI (kg/m^2)	23.27±3.33	22.92±3.11	23.28±3.34	0.114
Basal FSH (U/L)	6.31±1.82	6.36±1.93	6.31±1.81	0.683
Ovarian stimulation Protocol* (n)				0.173
GnRH agonist	3887	106	3781	
GnRH antagonist	38	2	36	
Mid-stimulation	12	1	11	
Insemination method* (n, %)				0.061
conventional IVF	3071(78.00)	93(3.03)	2978	
ICSI	866(22.00)	16(1.85)	850	
AH (n, %)				0.172
no	3462(37.51)	95(2.74)	3367	
yes	5767(62.49)	132(2.29)	5635	
Type of ET (n, %)				0.098
Fresh	3937(42.66)	109(2.77)	3828	
Frozen - thawed	5292(57.34)	118(2.23)	5174	
In *vitro* culture time (n, %)				0.040
Day 5	7897(85.57)	205(2.60)	7692	
Day 6	1332(14.43)	22(1.65)	1310	
Blastocyst stage (n, %)				0.589
1	4(0.04)	0	4	
2	72(0.78)	2(2.78)	70	

3	2898(31.40)	62(2.14)	2836	
4	5926(64.21)	154(2.60)	5772	
5	247(2.68)	8(3.24)	239	
6	82(0.89)	1(1.22)	81	
Inner cell mass grading (n, %)				0.038
A	2759(29.89)	82(2.97)	2677	
B	6470(70.11)	145(2.24)	6325	
Trophectoderm grading (n, %)				0.002
A	942(10.21)	32(3.40)	910	
B	5889(63.81)	157(2.67)	5732	
C	2398(25.98)	38(1.58)	2360	
Endometrial preparation protocols # (n, %)				0.52
NC	1826	44(2.41)	1782	
HRT	3466	74(2.14)	3392	

Note: Data are presented as mean ± standard deviation or number (percentage), unless otherwise indicated. BMI = body mass index; FSH = follicle-stimulating hormone; GnRH = gonadotropin-releasing hormone; IVF = in *vitro* fertilization; ICSI = intracytoplasmic sperm injection; ET = embryo transfer; NC = natural cycle; HRT = hormone replacement therapy; MZT = monozygotic twinning; * only of fresh ET. # only of frozen-thawed ET.

Table2 Multivariate analysis of the blastocyst morphology parameters on MZT

Variable	aOR	95%CI	*P* value
Maternal age	0.988	0953-1.024	0.501
Type of ET			
Fresh	ref		
Frozen - thawed	0.786	0.424-1.457	0.445
In *vitro* culture time			
Day 5	1.281	0.787-2.087	0.319
Day 6	ref		
AH			
Yes	1.201	0.630-2.287	0.578
No	ref		
Blastocyst stage			
1+2	ref		
3+4	0.889	0.216-3.666	0.871
5+6	1.125	0.235-5.385	0.883
ICM grading			
A	1.057	0.764-1.463	0.737
B	ref		
TE grading			
A	1.883	1.069-3.315	0.028
B	1.559	1.066-2.279	0.022
C	ref		

Note: AH = assist hatching; ICM = Inner cell mass; TE = trophectoderm; aOR = adjust odds ratio; CI = confidence interval; ref = reference

Figures

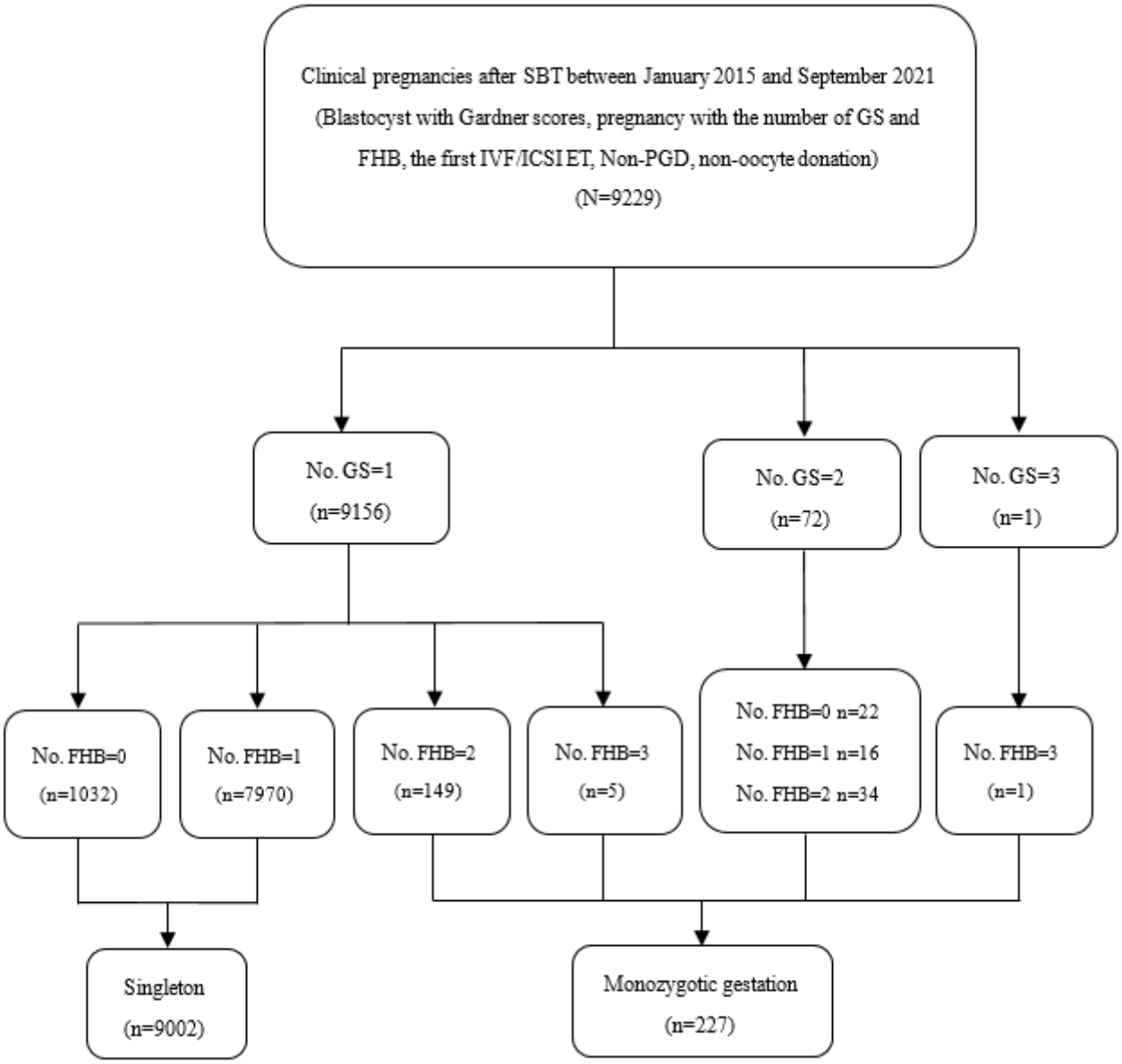

Figure 1

Flowchart of the retrospective cohort study. *Note*: SBT= single blastocyst transfer; GS= gestational sac; FHB= fetal heartbeat; No.= number of.; IVF= in *vitro* fertilization; ICSI= intracytoplasmic sperm injection; ET= embryo transfer; PGD= preimplantation genetic diagnosis

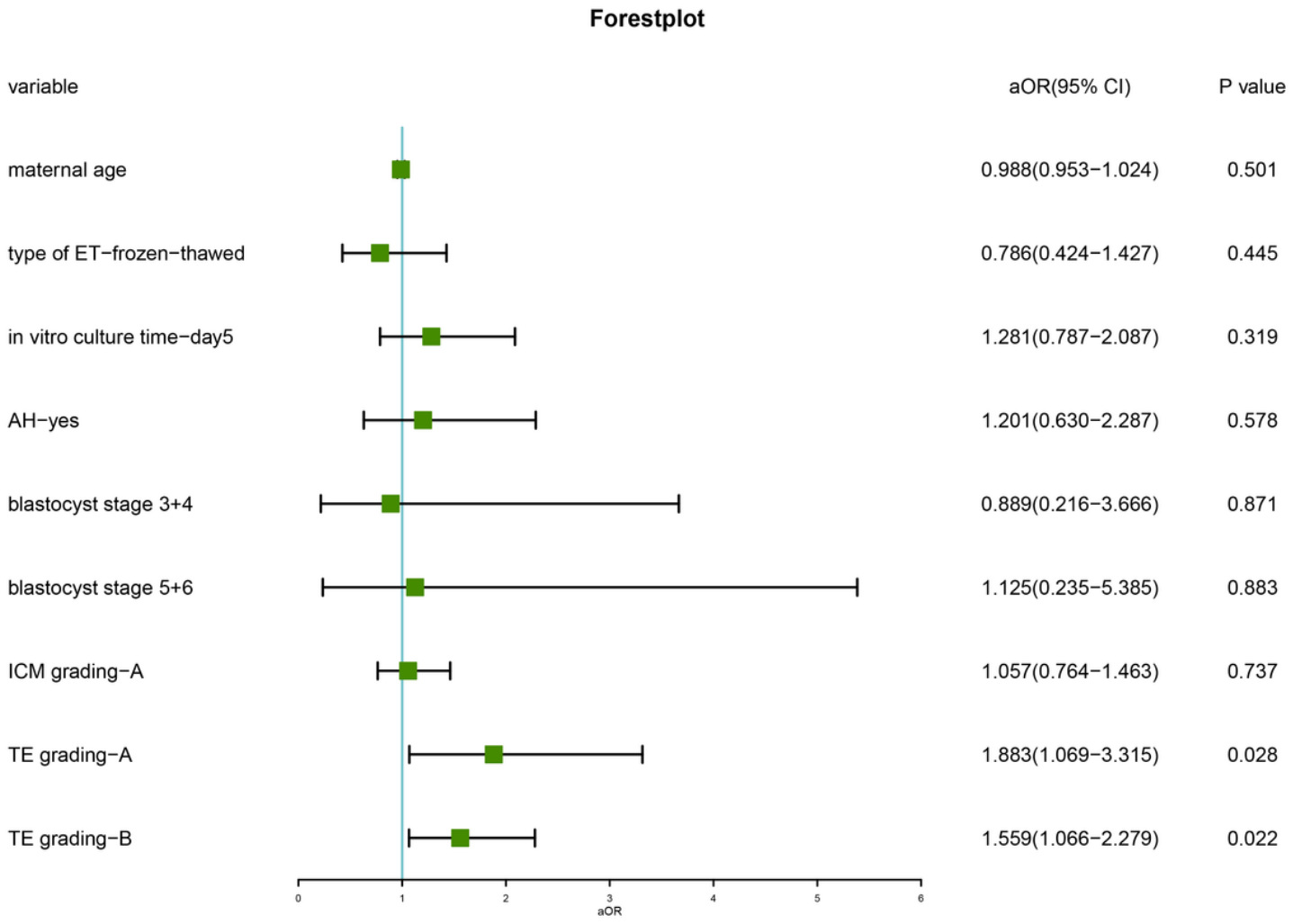

Figure 2

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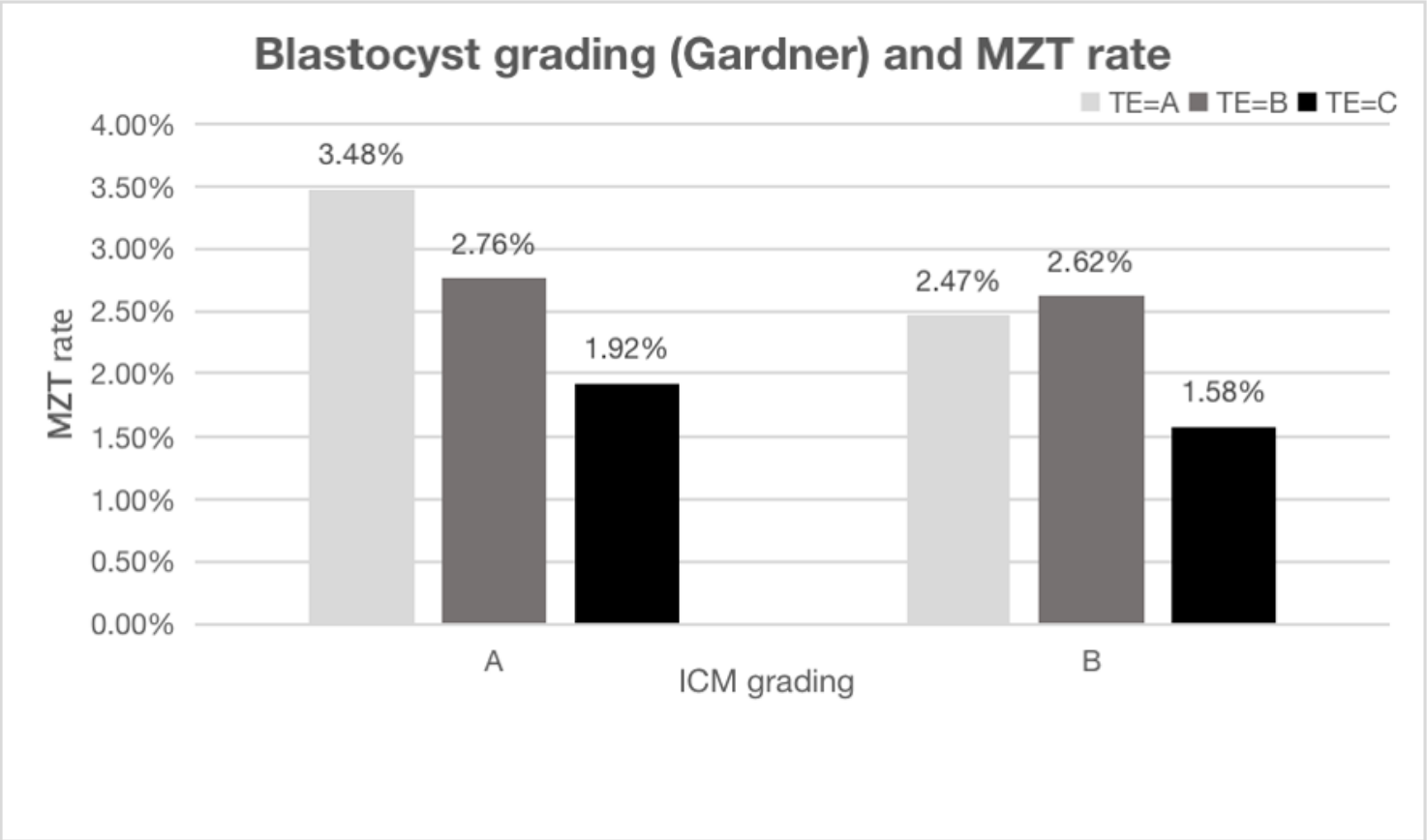

Figure 3

MZT rate of different ICM and TE grading of blastocysts based on the Gardner scoring system

Note: ICM = Inner cell mass; TE = trophectoderm; MZT = monozygotic twinning