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The effects of the day of trophectoderm biopsy and blastocyst grade on the clinical and neonatal outcomes of preimplantation genetic testing–frozen embryo transfer cycles

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Summary

This study analyzed the effects of the day of trophectoderm (TE) biopsy and blastocyst grade on clinical and neonatal outcomes. The results showed that the implantation and live birth rates of day 5 (D5) TE biopsy were significantly higher compared with those of D6 TE biopsy. The miscarriage rate of the former was lower than that of the latter, but there was no statistically significant difference. Higher quality blastocysts can achieve better implantation and live birth rates. Among good quality blastocysts, the implantation and live birth rates of D5 and D6 TE biopsy were not significantly different. Among fair quality and poor quality blastocysts, the implantation and live birth rates of D5 TE biopsy were significantly higher compared with those of D6 TE biopsy. Neither blastocyst grade nor the day of TE biopsy significantly affected the miscarriage rate. Neonatal outcomes, including newborn sex, gestational age, preterm birth, birth weight and low birth weight in the D5 and D6 TE biopsies were not significantly different. Both blastocyst grade and the day of TE biopsy must be considered at the same time when performing preimplantation genetic testing–frozen embryo transfer.

Introduction

Assisted reproductive technologies have helped thousands of infertile patients have children. Historically, more than one embryo is transferred into the uterus during the *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) process, which can lead to multiple pregnancy. Multiple pregnancy is not only dangerous for the mother, but also increases the incidence of miscarriage, premature birth and low birth weight (LBW) infants. Single embryo transfer is the most effective way to reduce the likelihood of a multiple pregnancy, and selective single blastocyst transfer can significantly reduce multiple pregnancy without reducing the pregnancy rate at this time (Sundhararaj *et al*., 2017; Kwek *et al*., 2018).

With the development of *in vitro* culture technology, normal fertilized oocytes can develop into viable blastocysts *in vitro* by day (D)5 or D6 after insemination. The most viable blastocyst is chosen for transfer to have a healthy live birth, which is beneficial for infertile patients. In the reported literature, there are inconsistencies regarding the influences of D5 and D6 blastocysts on the clinical outcomes of frozen embryo transfer (FET) cycles. A meta-analysis found that D5 and D6 cryopreserved blastocysts at the same developmental stage had similar clinical pregnancy and live birth rates (Sunkara *et al*., 2010). El-Toukhy *et al.* (2011) found that the rates of clinical pregnancy and live birth of high-grade blastocysts were not significantly different between D5 and D6 vitrified–warmed blastocysts. Kaye *et al.* (2017) reported that single high-quality D6 blastocyst transfer can obtain a similar clinical pregnancy rate to that of D5 blastocyst transfer in FET cycles.

However, Poulsen *et al.* (2017) reported that the implantation rate of D5 single blastocyst transfer was significantly higher compared with that of D6 single blastocyst transfer from fresh cycles. Several studies have shown that the rates of clinical pregnancy, implantation and live birth of D5 single blastocyst transfer were also significantly higher compared with those of D6 single blastocyst transfer in FET cycles (Ferreux *et al*., 2018; Sciorio *et al*., 2018, 2019; Tubbing *et al*., 2018). Two recent meta-analyses reported that the clinical pregnancy, implantation and live birth rates of D5 blastocyst transfer were significantly higher compared with those of D6 blastocyst transfer regardless of whether a fresh transfer or FET cycle was used (Bourdon *et al*., 2019; Li *et al*., 2020). These results may be related to the fact that the proportion of high-quality and euploid blastocysts on D5 was significantly higher compared with that on D6, which leads to better clinical outcomes (Minasi *et al*., 2016; Barash *et al*., 2017; Zhao *et al*., 2018). Therefore, the present study analyzed the effects

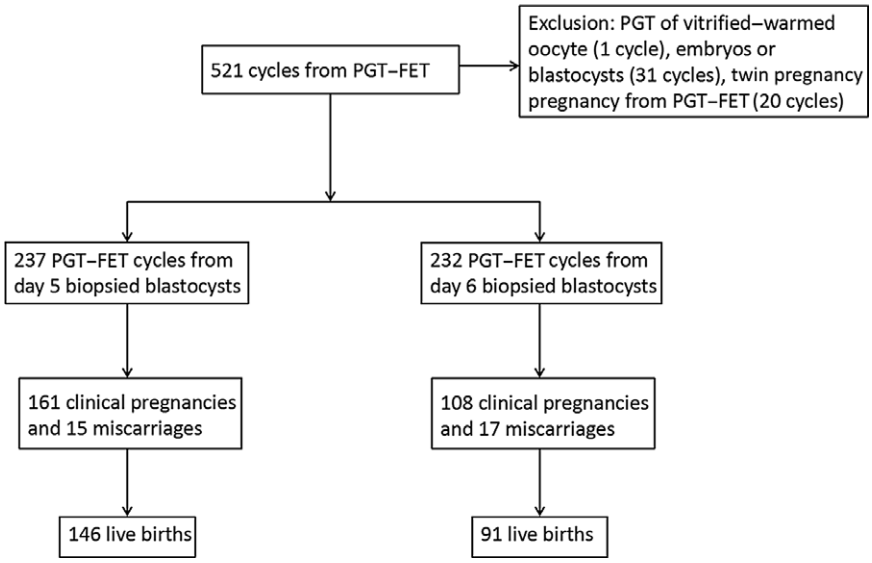

Figure 1. Flow diagram of the study

of the day of trophectoderm (TE) biopsy and blastocyst grade on the clinical and neonatal outcomes of preimplantation genetic testing–frozen embryo transfer (PGT–FET) cycles.

Materials and Methods

Patients

All infertile couples signed informed consent for PGT–FET treatment from January 2017 to December 2019 before participating in the present study. Our retrospective study consisted of the D5 TE biopsy group (D5) and the D6 TE biopsy group (D6). Patients with D5 or D6 blastocyst transfers were included in this study. Patients with PGT of vitrified–warmed oocytes, embryos or blastocysts, and twin-pregnancy cycles were excluded from this study (Fig. 1). Our retrospective study included 146 newborns from 237 transfer cycles in the D5 group and 91 newborns from 232 transfer cycles in the D6 group.

Insemination and embryo culture

Controlled ovarian hyperstimulation and oocyte pick-up were performed in accordance with the routine operation process of our IVF centre. Oocyte denudation was performed 3 h after oocyte pick-up. MII oocytes were inseminated by ICSI 1 h after oocyte denudation. The injected oocytes were cultured in separated G1 microdroplets in a humidified incubator. Oocyte fertilization was checked 16–18 h post-insemination. The fertilized oocytes containing two pronuclei continued to be cultured in G1 microdroplets until D3 post-insemination. A small hole with a diameter of 12 μm was drilled into the zona pellucida of the cleavage stage embryos by means of a laser on the D3 morning post-insemination so that the TE cells could herniate out of the hole for biopsy.

Blastocyst grading, TE biopsy, and biopsied blastocyst vitrification

The Gardner scoring system was used for blastocyst grading (Gardner and Schoolcraft, 1999). In our retrospective study, blastocysts with a score > BB, BB and < BB were considered good, fair and poor quality, respectively. TE biopsy was performed on D5 or D6 post-insemination. In total, 5–10 TE cells were cut using a laser and transferred into 200-μl PCR tubes for genetic analysis. A Vitrification Kit (KITAZATO) was used for vitrification of the biopsied blastocysts.

PGT procedure

The PGT procedure was performed with next-generation sequencing (NGS) on a MiSeqDx system (Illumina). Whole genome amplification (WGA) (SurePlex DNA Amplification System, Illumina), library construction (TG DNA Library Prep Kit, Illumina), sequencing (MiSeq^{TM} DX Reagent Kit v3, Illumina), and sequencing data analysis were performed in strict accordance with the manufacturer's instructions. The PGT cycles from monogenic disorders were diagnosed using NGS-based haplotyping, which was described in detail in our previously published literature (Chen *et al.*, 2016, 2017, 2019).

FET treatment

Preparation of the endometrium in FET cycles was performed by hormone replacement therapy (HRT), mild stimulation and gonadotropin (Gn) stimulation cycles. Warming of vitrified blastocysts was performed using a Thawing Kit (KITAZATO) on the morning of D6 progesterone administration. The vitrified–thawed blastocysts were transferred into the uterus 2 h after warming.

Definition

Clinical pregnancy, miscarriage, live birth, gestational age, preterm birth, and LBW were defined according to the reported literature (Zegers-Hochschild *et al.*, 2017).

Follow-up

A gestational sac with a fetal heartbeat scanned by ultrasound on D28 after blastocyst transfer indicated a clinical pregnancy. Data on neonatal outcomes, including date of birth, sex, birth weight and live birth, were obtained after birth.

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Statistical analysis

SPSS Statistics 22.0 software was used for data analysis. Independent samples Mann–Whitney *U*-test or Student's *t*-test was used to analyze female age, female body mass index (BMI), thickness of endometrium, gestational age and birth weight between the two groups. Data on endometrial preparation, categories of PGT, grade of transferred blastocysts, and the rates of implantation, miscarriage, live birth, infant sex, preterm birth and LBW were analyzed using the chi-squared (χ^2) test. Logistic regression analysis after adjusting for confounding factors (female age, female BMI, endometrial preparation, thickness of endometrium, categories of PGT) was used to analyze the association between the implantation and live birth rates and the day of TE biopsy and blastocyst grade. A *P*-value less than 0.05 means a significant difference.

Results

Female age, endometrial preparation, thickness of endometrium, and categories of PGT in the D5 TE biopsy group were not significantly different compared with that in the D6 TE biopsy group. Female BMI in the D5 TE biopsy group was significantly lower compared with that in the D6 TE biopsy group. The grade of transferred blastocysts in the D5 TE biopsy group was significantly higher compared with that in the D6 TE biopsy group, especially for good quality blastocysts (28.3% vs. 10.3%, $P < 0.001$, Table 1).

The implantation rate in the D5 TE biopsy group was significantly higher compared with that in the D6 TE biopsy group (67.9% vs. 46.6%, $P < 0.001$; Table 2). The odds ratio (OR) remained significant after adjusting for the day of TE biopsy (D5 vs. D6), female age, female BMI, endometrial preparation, thickness of endometrium, categories of PGT, and grade of transferred blastocysts [$P < 0.001$, adjusted OR (aOR) 2.2, 95% confidence interval (CI) 1.5–3.3; Table 3]. The implantation rate of good quality blastocysts was significantly higher than that of fair quality blastocysts (71.4% vs 59.0%, $P = 0.045$, Table 2; aOR 1.6, 95% CI 0.9–2.7; Table 3), and a similar result was obtained between good quality blastocysts vs. poor quality blastocysts (71.4% vs. 42.0%, $P < 0.001$, Table 2; aOR 2.6, 95% CI 1.4–4.9, Table 3) and fair quality blastocysts vs. poor quality blastocysts (59.0% vs. 42.0%, $P = 0.003$, Table 2; aOR 1.9, 95% CI 1.2–3.1, Table 3). Among good quality blastocysts, the implantation rate of the D5 blastocysts was not significantly different compared with that of the D6 blastocysts (73.1% vs. 66.7%, $P = 0.602$, Table 2; aOR 1.5, 95% CI 0.5–4.5, Table 3). However, among fair quality blastocysts or poor quality blastocysts, the implantation rate of the D5 blastocysts was significantly higher compared with that of the D6 blastocysts (67.2% vs 51.8%, $P = 0.013$, Table 2, aOR 2.1, 95% CI 1.2–3.5, Table 3; 62.2% vs 28.4%, $P < 0.001$, Table 2, aOR 3.7, 95% CI 1.6–8.7, Table 3).

The live birth rate of the D5 TE biopsy group was significantly higher compared with that of the D6 TE biopsy group (61.6% vs. 39.2%, $P < 0.001$, Table 2; aOR 2.4, 95% CI 1.6–3.6, Table 4). Similar to the implantation rate, there was a significant difference in the live birth rate between good quality blastocysts and poor quality blastocysts (59.3% vs. 39.3%, $P = 0.005$, Table 2; aOR 1.6, 95% CI 0.8–2.9, Table 4) and between fair quality blastocysts and poor quality blastocysts (52.3% vs. 39.3%, $P = 0.024$, Table 2; aOR 1.6, 95% CI 1.0–2.6, Table 4). The live birth rate of good quality and fair quality blastocysts was not significantly different (59.3% vs. 52.3%, $P = 0.273$, Table 2; aOR 1.2, 95% CI 0.7–2.0, Table 4). Among good quality blastocysts, the live birth rate of the D5 TE biopsy group was not significantly different compared with that of the D6 TE biopsy group (64.2% vs. 45.8%, $P = 0.148$, Table 2; aOR 2.1, 95% CI 0.8–5.8, Table 4). Among fair and poor quality blastocysts, the live birth rate of the D5 TE biopsy group was significantly higher compared with that of the D6 TE biopsy group (60.0% vs. 45.4%, $P = 0.020$, Table 2; aOR 2.0, 95% CI 1.2–3.3, Table 4; 62.2% vs. 23.9%, $P < 0.001$, Table 2; aOR 4.4, 95% CI 1.9–10.4, Table 4).

The miscarriage rates of the D5 TE biopsy and D6 TE biopsy groups were not significantly different (9.3% vs. 15.7%, $P = 0.126$, Table 2). At the same time, the miscarriage rate was not significantly affected by the grade of transferred blastocysts (Table 2). Moreover, the rates of infant sex, preterm birth and LBW, gestational age and birth weight of infants of the D5 TE biopsy group were not significantly different compared with that of the D6 TE biopsy group (Table 5).

Table 1. Maternal and cycle characteristics according to D5 and D6 trophectoderm biopsy

	D5	D6	*P*-value
Number of transfer cycles	237	232	
Female age (years)	30.3 ± 4.2	30.2 ± 4.2	0.888
Female BMI (kg/m^2)	22.5 ± 3.1	23.5 ± 3.1	<0.001
Endometrial preparation			0.188
HRT cycles	216 (91.1)[a]	199 (85.8)	
Mild stimulation cycles	13 (5.5)	20 (8.6)	
Gn stimulation cycles	8 (3.4)	13 (5.6)	
Thickness of endometrium (mm)	9.5 ± 1.4	9.7 ± 1.5	0.075
Categories of PGT			0.194
PGT-M	19 (8.0)	23 (9.9)	
PGT-A	56 (23.6)	69 (29.7)	
PGT-SR	162 (68.4)	140 (60.3)	
Grade of transferred blastocysts			<0.001
Good quality	67 (28.3)[b]	24 (10.3)[b]	
Fair quality	125 (52.7)	141 (60.8)	
Poor quality	45 (19.0)[c]	67 (28.9)[c]	

[a]Values in parenthesis are expressed in percentage.[b]<0.001.
[c]0.013.
BMI, body mass index; Gn, gonadotropin; HRT, hormone replacement therapy; PGT, preimplantation genetic testing; PGT-A, PGT for aneuploidies; PGT-M, PGT for monogenic disorders; PGT-SR, PGT for chromosome structural rearrangements.

Discussion

The present study showed that the PGT–FET cycles involving better-grade blastocysts obtained higher implantation and live birth rates. The implantation and live birth rates of D5 TE biopsy were superior to those of D6 TE biopsy for similarly graded blastocysts. The blastocyst grade and the day of TE biopsy did not significantly affect the miscarriage rate. The neonatal outcomes, including sex, gestational age, preterm birth, birth weight, and LBW of newborns, were not significantly different between D5 TE biopsy and D6 TE biopsy.

Minasi *et al.* (2016) reported that the euploid rate of blastocysts with top-quality ICM was significantly higher than that of blastocysts with poor quality ICM, and the similar results were obtained

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Table 2. Clinical outcomes according to D5 and D6 trophectoderm biopsy

	Category	Implantation rate (%)	*P*-value	Miscarriage rate (%)	*P*-value	Live birth rate (%)	*P*-value
Day of TE biopsy	5	67.9 (161/237)	Ref.	9.3 (15/161)	Ref.	61.6 (146/237)	Ref.
	6	46.6 (108/232)	<0.001	15.7 (17/108)	0.126	39.2 (91/232)	<0.001
Blastocyst grade	Good	71.4 (65/91)	Ref.	16.9 (11/65)	Ref.	59.3 (54/91)	Ref.
	Fair	59.0 (157/266)[a]	0.045	11.5 (18/157)	0.280	52.3 (139/266)[b]	0.273
	Poor	42.0 (47/112)[a]	<0.001	6.4 (3/47)	0.147	39.3 (44/112)[b]	0.005
Combined criteria	D5 good	73.1 (49/67)	Ref.	12.2 (6/49)	Ref.	64.2 (43/67)	Ref.
	D6 good	66.7 (16/24)	0.602	31.3 (5/16)	0.121	45.8 (11/24)	0.148
	D5 fair	67.2 (84/125)	Ref.	10.7 (9/84)	Ref.	60.0 (75/125)	Ref.
	D6 fair	51.8 (73/141)	0.013	12.3 (9/73)	0.805	45.4 (64/141)	0.020
	D5 poor	62.2 (28/45)	Ref.	0.0(0/28)	Ref.	62.2 (28/45)	Ref.
	D6 poor	28.4 (19/67)	<0.001	15.8 (3/19)	0.060	23.9 (16/67)	<0.001

a = 0.003, b = 0.024.
TE, trophectoderm.

Table 3. Results of logistic regression analysis of implantation rate after adjusting for confounding factors according to maternal and cycle characteristics

	aOR	95% CI	*P*-value
D5 vs. D6	2.2	1.5–3.3	<0.001
Grade of transferred blastocyst			
Good vs. Fair	1.6	0.9–2.7	0.102
Good vs. Poor	2.6	1.4–4.9	0.003
Fair vs. Poor	1.9	1.2–3.1	0.005
D5 vs. D6 (good)	1.5	0.5–4.5	0.476
D5 vs. D6 (fair)	2.1	1.2–3.5	0.006
D5 vs. D6 (poor)	3.7	1.6–8.7	0.003

aOR, adjusted odds ratio; CI, confidence interval.

Table 4. Results of logistic regression analysis of live birth rate according to maternal and cycle characteristics

	aOR	95% CI	*P*-value
D5 vs. D6	2.4	1.6–3.6	<0.001
Grade of transferred blastocyst			
Good vs. Fair	1.2	0.7–2.0	0.523
Good vs. Poor	1.6	0.8–2.9	0.166
Fair vs. Poor	1.6	1.0–2.6	0.037
D5 vs. D6 (good)	2.1	0.8–5.8	0.140
D5 vs. D6 (fair)	2.0	1.2–3.3	0.008
D5 vs. D6 (poor)	4.4	1.9–10.4	0.001

aOR, adjusted odds ratio; CI, confidence interval.

Table 5. Neonatal outcomes according to D5 and D6 trophectoderm biopsy

	D5	D6	*P*-value
Number of live births	146	91	—
Infant sex			0.589
Boys	82 (56.2)[a]	55 (60.4)	
Girls	64 (43.8)	36 (39.6)	
Gestational age (weeks)	39.2 ± 1.4	38.9 ± 1.5	0.185
Preterm birth	8 (5.5)	12 (13.2)	0.053
Birth weight (g)	3463.1 ± 486.8	3498.6 ± 541.6	0.602
LBW (<2500 g)	4 (2.7)	2 (2.2)	1.000

[a]Values in parentheses are expressed in percentage.
LBW, low birthweight.

for blastocysts with high-quality TE compared with blastocysts with poor quality TE; that is, a higher blastocyst grade will lead to a higher euploid rate for blastocysts. Ozgur *et al.* (2019) found that the live birth rate was not significantly different between single best-scoring and unknown-ploidy blastocyst transfer and single best euploid blastocyst transfer from infertile patients who were no more than 35 years old. Viñals Gonzalez *et al.* (2019) reported that the euploid rate of blastocysts was associated with blastocyst grade, while the rates of implantation and live birth were not significantly affected by blastocyst morphology in patients of advanced maternal age who had preimplantation genetic testing for aneuploidy cycles. However, our retrospective study found that the rates of implantation and live birth were related to blastocyst grade, and higher quality blastocysts could obtain better implantation and live birth rates, which was consistent with two other studies (Zhao *et al.*, 2018; Irani *et al.*, 2018).

In addition to blastocyst morphology, the speed of blastocyst development significantly affected clinical outcomes. Franasiak *et al.* (2018) reported that the sustained implantation rate in slowly blastulating embryos on D5 was significantly lower than that in normally blastulating embryos regardless of age in fresh cycles. At the same time, D6 fresh embryo transfer also had a significantly lower sustained implantation rate in slowly blastulating embryos on D5 than that in normally blastulating embryos, despite the blastocyst morphology grade being equivalent when the embryo transferred. The sustained implantation rate in the FET cycle was not significantly different between slowly blastulating embryos and normally blastulating embryos. The above results suggest that the different sustained implantation rates in the fresh cycle

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between slowly blastulating embryos and normally blastulating embryos on D5 are due to desynchrony between the embryo and endometrium. However, two new meta-analyses reported that the implantation, clinical and live birth rates of D5 blastocyst transfer were significantly higher compared with those of D6 blastocyst transfer regardless of whether a fresh and frozen transfer cycle was used (Bourdon *et al*., 2019; Li *et al*., 2020). This may be related to the euploidy rate being significantly higher among D5 blastocysts compared with among D6 blastocysts, which can result in better clinical outcomes. Our retrospective study showed that the implantation and live birth rates of D5 euploid blastocyst transfer were significantly higher compared with those of D6 euploid blastocyst transfer from PGT–FET cycles, which was similar to the reported study (Irani *et al*., 2018).

It has been reported that the clinical pregnancy and live birth rates of high-quality and high-grade blastocysts were comparable among D5 and D6 vitrified–warmed blastocysts (El-Toukhy *et al*., 2011; Kaye *et al*., 2017). Similar to the reports presented above, the implantation and live birth rates from good quality blastocysts were not significantly different between D5 and D6 euploid blastocyst transfers in our retrospective study. The present study also found that the implantation and live birth rates of the D5 euploid blastocyst transfers were higher than those of the D6 euploid blastocyst transfers for similarly graded euploid blastocysts, which was consistent with Irani's study (Irani *et al*., 2018). There may also be some embryonic intrinsic factors, such as RNA expression, metabolic differences or epigenetic differences resulting in superior clinical outcomes for D5 euploid blastocysts compared with D6 euploid blastocysts.

Aneuploidy of blastocysts is related to miscarriage. It has been reported that there was no significant difference in the miscarriage rates between D5 and D6 euploid blastocyst transfer cycles (Hernandez-Nieto *et al*., 2019). Similar to the literature reported above, the present study showed that blastocyst morphology grade and development speed did not significantly affect the miscarriage rate of either the D5 or D6 euploid blastocyst transfer cycle. A meta-analysis showed that there was no significant difference in perinatal outcomes between D5 and D6 blastocyst transfer cycles, while birth weight was associated with extended *in vitro* culture (Zeng *et al*., 2020). Our study showed that there was no significant difference in neonatal outcomes between the two groups.

In conclusion, both blastocyst grade and the day of TE biopsy should be considered simultaneously for euploid blastocyst transfer so that better clinical and neonatal outcomes can be obtained.

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Conflicts of interest. The authors declared no potential conflicts of interest.

Ethics statements. This retrospective study was approved by the Ethics Committee of Nanjing Drum Tower Hospital affiliated with Nanjing University Medical School (No. 2020–019).

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