

Clinical outcomes for Day 3 double cleavage-stage embryo transfers versus Day 5 or 6 single blastocyst transfer in frozen–thawed cycles: a retrospective comparative analysis

Journal of International Medical Research

49(12) 1–12

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DOI: 10.1177/03000605211062461

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Abstract

Objective: This study aimed to compare the clinical outcomes for transfer of Day 3 (D3) double cleavage-stage embryos and Day 5/6 (D5/6) single blastocysts in the frozen embryo transfer (FET) cycle to formulate a more appropriate embryo transplantation strategy.

Methods: We retrospectively analyzed 609 FET cycles from 518 women from April 2017 to March 2021. All FETs were assigned to the D3-DET group (transfer of a Day 3 double cleavage-stage embryo), D5-SBT group (transfer of a Day 5 single blastocyst), or D6-SBT group (transfer of a Day 6 single blastocyst). Clinical outcomes were comparatively analyzed.

Results: There were no significant differences in the biochemical pregnancy rate, clinical pregnancy rate, or ongoing pregnancy rate between the D3-DET and D5-SBT groups, but these rates in the two groups were all significantly higher compared with those in the D6-SBT group. The implantation rate in the D5-SBT group was significantly higher than that in the D3-DET group.

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The twin pregnancy rate in the D5-SBT and D6-SBT groups was significantly lower than that in the D3-DET group.

Conclusion: This study suggests that D5-SBT is the preferred option for transplantation. D6-SBT reduces the pregnancy rate, making it a more cautious choice for transfer of such embryos.

Keywords

Frozen embryo transfer, double cleavage-stage embryo transfer, single blastocyst transfer, implantation rate, twin pregnancy rate, clinical pregnancy rate

Date received: 27 May 2021; accepted: 18 October 2021

Introduction

Transfer of multiple embryos may increase the possibility of a live birth in the practice of *in vitro* fertilization and embryo-transfer (IVF-ET).¹ Additionally, there is an increased risk of multiple pregnancies in IVF-ET, accompanied by a series of severe maternal and neonatal complications. These complications include pregnancy-induced hypertension, twin transfusion syndrome, fetal presentation and an abnormal fetal position, premature delivery and rupture of the membranes, placental abruption, polyhydramnios, amniotic fluid embolism, uterine inertia, postpartum hemorrhage, fetal intrauterine growth retardation, low birth weight infants, abortion, and fetal malformation.²⁻⁵ Transfer of a single embryo is the most direct approach for a reduction in multiple pregnancies. If the number of embryos transferred is consistent, good-quality blastocysts in a prolonged *in vitro* culture of embryos are associated with higher implantation and clinical pregnancy rates compared with those in cleavage-stage embryos.⁶⁻¹¹ In past years, single blastocyst transfer has been applied by an increasing amount of reproductive centers. However, there is a risk of failed blastulation of some

or all embryos in the process of blastocyst culture, which results in a reduction in the availability of embryos and even cancellation of the transfer cycle.¹² This situation cannot be accepted by patients with high expectations of assisted reproduction. Additionally, embryos vary in their development rate (potential) in *in vitro* culture, leading to the formation of usable blastocysts on different development days (Day 5/6). Whether single blastocyst transfer generates better clinical outcomes than double cleavage-stage embryo transfer remains controversial,^{7,12} and there is no international guideline or consensus on the embryo transfer strategy.

Our center pre-freezes two to four high-quality cleavage-stage embryos on Day 3 (D3) of embryo development to ensure the availability of embryos. The rest of the embryos are collected for blastocyst formation, and the usable blastocysts that form on D5 and D6 are frozen for further transfer analysis. This study aimed to investigate the clinical outcomes for transfer of D3 double cleavage-stage embryos versus D5/D6 single blastocysts in the frozen embryo transfer (FET) cycle. We hope to provide a reference for a more appropriate strategy for embryo transfer.

Materials and methods

Subjects

This retrospective analysis included 609 FET cycles from 518 women who visited the Center for Reproductive Medicine of The Second Affiliated Hospital, Zhejiang University School of Medicine between April 2017 and March 2021. All cycles were assigned into the D3-DET group (transfer of Day 3 double cleavage-stage embryos), D5-SBT group (transfer of a Day 5 single blastocyst), and D6-SBT group (transfer of a Day 6 single blastocyst). The inclusion criteria were as follows: (1) women aged ≤ 37 years old; (2) secondary infertility, length of infertility < 5 years; (3) endometrial thickness ≥ 7 mm and

< 14 mm at embryo transfer; and (4) D3-DET (grade I/II), D5-SBT, or D6-SBT (at least grade 3BC or 3CB). Exclusion criteria were as follows: (1) recurrent implantation loss (no clinical pregnancy in more than three times of transfer); (2) endocrine diseases, such as hyperprolactinemia and hyperthyroidism/hypothyroidism; (3) uterine cavity lesions or abnormalities, such as uterine malformation, adenomyosis, uterine cavity effusion, and a history of intrauterine adhesion surgery; (4) endometrial thickness < 7 mm; (5) an abnormal chromosome in the husband or wife; and (6) embryos or blastocysts that did not fully survive after thawing (partial blastomere degeneration or insufficient dilation of the blastocyst cavity) (Figure 1).

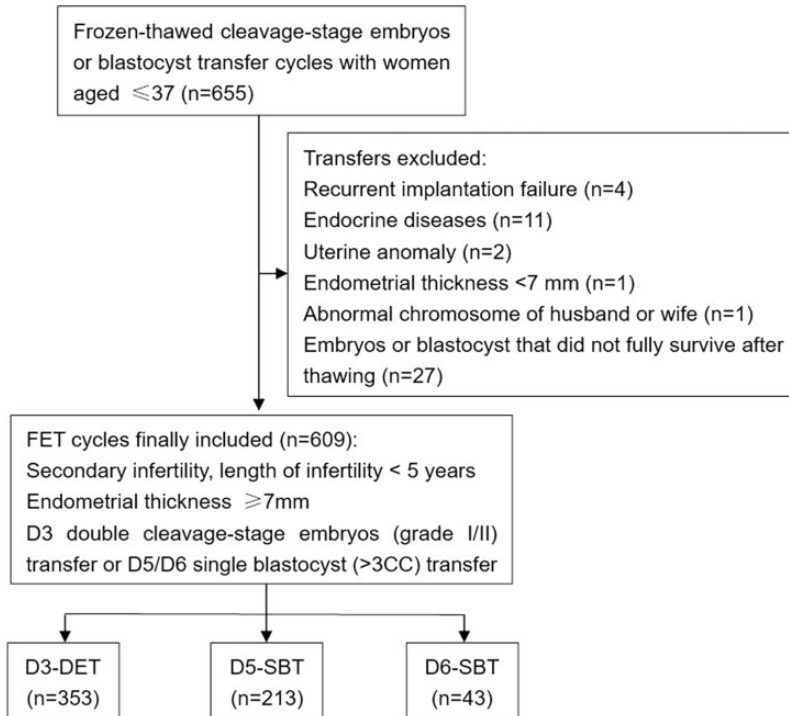


Figure 1. Flow chart of inclusion and exclusion criteria of FET cycles and division into the three groups FET, frozen embryo transfer; D3-DET, transfer of a Day 3 double cleavage-stage embryo; D5-SBT, transfer of a Day 5 single blastocyst; D6-SBT, transfer of a Day 6 single blastocyst.

Ethical approval

This study was approved by the ethics committee of The Second Affiliated Hospital of Zhejiang University (reference no. 20170209) on 3 March 2017. All subjects along with their families were informed and provided written informed consent. All of the patients' details have been de-identified. The reporting of this study conforms to the STROBE guidelines.¹³

Controlled superovulation

An appropriate antagonist or agonist protocol was selected for ovulation induction by referring to the patient's age, anti-Müllerian hormone concentrations, basal hormone concentrations, and basal sinus follicle count. If the diameter of follicles was ≥ 18 mm and the number was \geq three, human chorionic gonadotrophin (hCG) (6500–8000 IU) was administered by intramuscular injection that night, and transvaginal B-ultrasound-guided oocyte retrieval was performed in 36 to 37 hours.

Gamete processing and embryo culture

Oocyte–corona cumulus complexes, which were searched for and collected by stereomicroscopy, were equilibrated in G-IVF (IVF medium; Vitrolife, Gothenburg, Sweden) at 37°C with 6% CO₂ for 3 to 4 hours. Spermatozoa were collected by density gradient centrifugation or the swim-up technique. Routine IVF or intracytoplasmic sperm injection was performed under the following conditions: 1) severe oligospermia, asthenospermia, or teratozoospermia (sperm density $< 5 \times 10^6$ /mL, sperm motility $< 10\%$, or sperm normal morphology rate $< 1\%$); 2) the previous IVF was not successful or the fertilization rate was $< 30\%$; 3) patients had sperm obtained through percutaneous epididymal sperm aspiration or testicular sperm aspiration due to azoospermia; 4) sperm without an

acrosome or an abnormal acrosome, such as round head sperm; and 5) immune infertility and failure of conventional IVF. Routine IVF or intracytoplasmic sperm injection was carried out at 39 to 41 hours after hCG administration, and the day of fertilization was defined as Day 0. The morphology and number of pronuclei were observed under an inverted microscope after 16 to 18 hours of fertilization (Day 1). Normal fertilization was defined as two pronuclei and two polar bodies. Fertilized oocytes were transferred to G-1 (cleavage-stage medium; Vitrolife) by microdroplets and grown in a hypoxic incubator composed of 5% O₂, 6% CO₂, and 89% N₂ for a further 48 hours. Embryos on Day 3 were scored, and two to four high-quality grade I/II embryos were selected and frozen. The rest the cleavage-stage embryos were transferred to G-2 (blastocyst culture medium; Vitrolife) by microdroplets and placed in a hypoxic incubator for further culture. On Day 5, embryos grew for another day in the presence of morula or stages 1 to 2 blastocysts, while blastocysts meeting usable standards were collected for vitrification. Blastocysts on Day 6 were evaluated and the usable ones were frozen (blastocysts that developed from post-thawed cleavage embryos were excluded in our study).

Embryo scoring

Cleavage-stage embryo grading was as follows: grade I, seven to nine blastomeres, with a uniform size, regular morphology, complete zona pellucida, homogenous and clear cytoplasm with no particles, and fragmentation events $< 10\%$; grade II, blastomeres \geq six, with a basically uniform size, particles present in the cytoplasm, and fragmentation events of 10% to 25%; and grade III, blastomeres \leq five, with an evident uneven size, irregular morphology, coarse particles in the cytoplasm, and fragmentation events $> 25\%$. Blastocyst staging

was performed in accordance with the Gardner standard.¹⁴ Stage 2 and above were defined as blastocyst formation. Usable blastocysts were defined as the presence of \geq stage 3 blastocyst dilation, and \geq grade B inner cell mass or trophoblast cells. High-quality blastocysts were considered as \geq stage 3 blastocyst dilation, and \geq grade B inner cell mass and trophoblast cells.

Freezing and thawing of cleavage-stage embryos and blastocysts

All embryos and blastocysts were vitrified and thawed¹⁵ with the Vitrification Kit, Thawing Kit, and Crytop carrier (Kitazato, Shizuoka, Japan). The blastocysts required laser shrinkage before freezing to discharge intracellular fluid. Cleavage-stage embryos and blastocysts were exposed to equilibrium solution at room temperature for 10 and 15 minutes, respectively, transferred into vitrification freezing solution for dehydration, and then loaded to the Crytop carrier. The carrier was then immediately put into liquid nitrogen, with the total procedure taking no more than 1 minute. When embryos and blastocysts were resuscitated, the carrier was taken out from the liquid nitrogen (-196°C), and then immediately immersed into thawing solution (37°C , 1 minute). Embryos and blastocysts were separately exposed to dilution solution for 3 minutes, followed by washing solution 1 (5 minutes) and washing solution 2 (1 minute) in sequence, and finally transferred into G-2 solution (Vitrolife) for further transfer.

Endometrial preparation, embryo transfer protocol, and luteal support

Endometrial preparation was performed by artificially adjusting the endometrium to the cleavage stage or the transfer window of blastocysts (D5/D6 blastocysts were

matched with D5 endometrium). FETs were performed using two artificial cycle regimens sequentially prepared by exogenous estrogen and progesterone hormones. Most artificial cycle protocols mimicked the natural cycle by using 2 mg 2/day estradiol valerate from the second to fifth days of the menstrual cycle, and 2 mg 1/day estradiol valerate and 0.5 mg 1/day 17β -estradiol after the end of the menstrual period. Another artificial cycle protocol was performed by providing 4 to 8 mg/day estradiol valerate from the second to fifth days of the menstrual cycle. If the endometrium reached 7 mm and serum estradiol concentrations reached 800 pmol/L, 60 mg/day of progesterone and 20 mg/day of dydrogesterone were used for transformation of the endometrium.

The embryo transfer protocol was as follows. If women had both cleavage embryos and blastocysts available, two D3 cleavage-stage embryos were routinely transferred first. If women were not pregnant after transferring two D3 cleavage-stage embryos or required re-transplantation after successful delivery, a D5 or D6 single blastocyst with the best morphological evaluation was selected for use. If women only had one D3 cleavage embryo frozen, but also had D5/D6 blastocysts available, a single D5/D6 blastocyst was transferred first. If women voluntarily asked for a single embryo transfer owing to economic (support cost) or family (already had one child) reasons, a single D5/D6 blastocyst was transferred first. If women had a cesarean section before the procedure or had problems related to the uterine cavity, a single D5/D6 blastocyst was transferred first. This protocol was in accordance with our center's standard operating procedure. Thawed blastocysts were incubated in G-2 solution for 3 to 4 hours to ensure survival for transfer, while thawed cleavage-stage embryos were grown in G-2 solution overnight.

Luteal support included estrogen and progesterone supplementation, and it was maintained until 10 weeks of pregnancy if pregnancy was established.

Outcome indicators

Serum hCG concentrations were measured 14 days after transplantation, and a biochemical pregnancy was established if hCG concentrations were >50 IU/L. Clinical pregnancy was established if the gestational sac and fetal heart beat were observed in a transvaginal ultrasound examination 4 to 5 weeks after transplantation. The number of gestational sacs was defined as the number of embryo implantations, and multiple pregnancies were established by the presence of two or more gestational sacs. An ectopic pregnancy was defined by the location of the gestational sac (outside the uterine cavity) under ultrasound or laparoscopy. Pregnancy loss 12 weeks before a clinical pregnancy was determined as early miscarriage. A pregnancy for longer than 12 weeks was defined as an ongoing pregnancy.

The biochemical pregnancy rate was calculated as follows: (number of biochemical pregnancies/number of transfer cycles) \times 100%. The clinical pregnancy rate was calculated as follows: (number of clinical pregnancies/number of transfer cycles) \times 100%. The embryo implantation rate (per thawing) was calculated as follows: (number of implanted embryos/number of thawed embryos) \times 100%. The embryo implantation rate (per transfer) was calculated as follows: (number of implanted embryos/number of transferred embryos) \times 100%. The ongoing pregnancy rate was calculated as follows: (number of ongoing pregnancy cycles/number of transfer cycles) \times 100%. The twin pregnancy rate was calculated as follows: (number of twin pregnancies/number of clinical pregnancies) \times 100%. The ectopic pregnancy rate was calculated

as follows: (number of ectopic pregnancies/number of clinical pregnancies) \times 100%. The early miscarriage rate was calculated as follows: (number of early miscarriages/number of clinical pregnancies) \times 100%.

Statistical methods

IBM SPSS 22.0 software (IBM Corp., Armonk, NY, USA) was used for data processing. Measurement data conformed to a normal distribution, and data shown as mean \pm standard deviation were analyzed by the *t*-test for between-group comparisons. Enumeration data (%) were compared by the χ^2 test or Fisher's exact probability method for comparisons. The hypothesis test was two-sided, and $P < 0.05$ was considered statistically significant.

Results

Basic characteristics

A total of 609 FET cycles were collected, and we studied 353 women with D3-DET, 213 with D5-SBT, and 43 with D6-SBT (Figure 1). There were no significant differences in age, body mass index (BMI), length of infertility, anti-Müllerian hormone concentrations, ovarian stimulation protocol (antagonist or agonist), average number of oocytes retrieved, fertilization method (intracytoplasmic sperm injection or IVF), or endometrial thickness at the embryo transfer among the three groups (Table 1).

Embryo resuscitation after thawing and implantation after transplantation

Of the 609 FET cycles, 1002 thawed embryos were obtained and 962 survived after thawing. The survival rate in the D3-DET group was 95.9%, and that in the D5-SBT and D6-SBT groups was 96.4% and 95.6%,

Table 1. Baseline characteristics of the patients in the three groups.

	D3-DET group (n = 353)	D5-SBT group (n = 213)	D6-SBT group (n = 43)	P value
Age (years)	31.3 ± 4.7	31.5 ± 4.2	30.7 ± 4.0	0.473
BMI (kg/m ²)	22.3 ± 9.8	21.8 ± 3.7	21.8 ± 3.1	0.730
Length of infertility (years)	2.9 ± 2.1	2.9 ± 2.1	2.4 ± 1.2	0.295
AMH (ng/mL)	3.93 ± 3.31	4.18 ± 3.42	4.14 ± 2.56	0.675
Ovarian stimulation protocol				
Antagonist protocol	230/353 (65.2%)	137/213 (64.3%)	22/43 (51.2%)	0.194
Agonist protocol	123/353 (34.8%)	76/213 (35.7%)	21/43 (48.8)	0.194
No. of oocytes retrieved	10.3 ± 0.6	11.3 ± 5.9	10.9 ± 5.7	0.130
Fertilization method				
ICSI	80/353 (22.7%)	42/213 (19.7%)	7/43 (16.3%)	0.508
IVF	273/353 (77.3%)	171/213 (80.3%)	36/43 (83.7%)	0.508
Endometrial thickness (mm)	10.3 ± 1.3	10.5 ± 1.5	10.1 ± 1.6	0.118

Data are presented as mean ± standard deviation or n (%).

D3-DET, transfer of a Day 3 double cleavage-stage embryo; D5-SBT, transfer of a Day 5 single blastocyst; D6-SBT, transfer of a Day 6 single blastocyst; BMI, body mass index; AMH, anti-Müllerian hormone; ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization.

respectively, with no significance difference among the three groups. All embryos that survived were taken for transfer. There was no significant difference in the rate of high-quality blastocysts between the D5-SBT and D6-SBT groups ($P=0.062$). The implantation/thawed rate in the D3-DET and D6-SBT groups was significantly lower than that in the D5-SBT group (relative risk [RR] 0.738, 95% confidence interval [CI]: 0.632–0.861, $P<0.001$; RR 0.466, 95% CI: 0.274–0.790, $P=0.001$, respectively). Similarly, the implantation/transfer rate in the D3-DET and D6-SBT groups was lower than that in the D5-SBT group (RR 0.741, 95% CI: 0.637–0.863, $P<0.001$; RR 0.470, 95% CI: 0.278–0.794, $P=0.001$, respectively) (Table 2).

Clinical outcomes

The biochemical pregnancy rate, clinical pregnancy rate, and ongoing pregnancy rate were not significantly different between the D3-DET and D5-SBT groups. However, these variables were significantly

higher in the D3-DET and D5-SBT groups than those in the D6-SBT group (all $P<0.05$). There was one case of monozygotic twins in the D5-SBT group and no cases were observed in the D6-SBT group, which were significantly lower than that in the D3-DET group ($P<0.001$). There was no significant difference in the early miscarriage rate or ectopic pregnancy rate among the three groups (Table 3).

By the end of May 2021, newborns from 166 D3 cleavage-stage embryos, 64 D5 blastocysts, and 4 D6 blastocysts were delivered without any visible defects, such as major structural malformations. The D3-DET group had a significantly higher risk of preterm delivery (27.1% [45/166] vs. 9.4% [6/64]), mean lower live birth weight (2.95 ± 0.65 vs. 3.35 ± 0.61 kg), and mean gestational age at delivery (37.6 ± 4.1 vs. 38.3 ± 2.4 weeks) compared with those in the D5-SBT group (all $P<0.05$). Four neonates born in the D6-SBT group did not have premature delivery, and the mean live birth weight (3.34 ± 0.28 kg) and gestational age (38.9 ± 1.3 weeks) were within the

Table 2. Comparison of the embryo or blastocyst outcome in the three groups.

	D3-DET group	D5-SBT group	D6-SBT group	P value
Thawed (n)	736	221	45	
Survived (n/N, %)	706/736 (95.9)	213/221(96.4)	43/45 (95.6)	0.867
Transferred (n)	706	213	43	
Mean number of embryos thawed/transfer	2.08 ± 0.34	1.04 ± 0.19	1.05 ± 0.21	<0.001
High-quality blastocysts transferred (n/N, %)	–	127/213 (59.6)	19/43 (44.2)	0.062
Implantation/ thawed (n/N, %)	285/736 (38.7) ^a	116/221 (52.5)	11/45 (24.4) ^a	<0.001
RR (95% CI)	0.738 (0.632–0.861)	Reference	0.466 (0.274–0.790)	
Implantation/ transferred (n/N, %)	285/706 (40.4) ^a	116/213 (54.5)	11/43 (25.6) ^a	<0.001
RR (95% CI)	0.741 (0.637–0.863)	Reference	0.470(0.278–0.794)	

Data are presented as mean ± standard deviation, n, n/N (%), or the 95% CI.

“Survived” means no blastomere degeneration (cleavage-stage embryo) or the blastocyst cavity was re-expanded (blastocyst). “High-quality blastocyst” means that the blastocysts reached 3BB or better quality. “n/N” indicates numerator/denominator.

^aP < 0.05 compared with the D5-SBT group.

D3-DET, transfer of a Day 3 double cleavage-stage embryo; D5-SBT, transfer of a Day 5 single blastocyst; D6-SBT, transfer of a Day 6 single blastocyst; RR, relative risk (compared with the D5-SBT group); CI, confidence interval.

Table 3. Comparison of clinical outcomes after embryo transfer in the three groups.

	D3-DET	D5-SBT group	D6-SBT group	P value
Biochemical pregnancy rate	232/353 (65.7) ^a	141/213 (66.2) ^a	19/43 (44.2)	0.016
Clinical pregnancy rate	203/353 (57.5) ^a	116/213 (54.5) ^a	11/43 (25.6)	<0.001
Ongoing pregnancy rate	180/353 (51.0) ^a	106/213 (49.8) ^a	6/43 (14.0)	<0.001
Twin pregnancy rate	82/203 (40.4)	1/116 (0.9) ^b	0/11 (0) ^b	<0.001
Ectopic pregnancy rate	9/203 (4.4)	1/116 (0.9)	1/11 (9.1)	0.088
Early miscarriage rate	23/203 (11.3)	10/116 (8.6)	3/11 (27.3)	0.151

Data are presented as n/N (%). “n/N” indicates numerator/denominator.

^aP < 0.05 compared with the D6-SBT group; ^bP < 0.05 compared with the D3-DET group.

D3-DET, transfer of a Day 3 double cleavage-stage embryo; D5-SBT, transfer of a Day 5 single blastocyst; D6-SBT, transfer of a Day 6 single blastocyst.

normal range. The sex ratio of newborns was 80:86 (0.93:1) in the D3-DET group, 34:27 (1.26:1) in the D5-SBT group, and 0:4 in the D6-SBT group (all four newborns were girls).

Discussion

This study suggests that compared with D3-DET, D5-SBT is the preferred option

for transplantation. IVF-ET technology is carried out with the requirement of an equilibrated clinical pregnancy, but pregnancy complications can occur when two or more cleavage-stage embryos are transferred. Cleavage-stage embryos on D3 in the FET cycle can produce a large number of usable embryos because there is a low risk of external exposure owing to a short *in vitro* culture time. However, the

high-quality embryos formed by D3 still have a frequency of chromosome aneuploidy of approximately 60%.¹⁶ This leads to a persistent low clinical pregnancy rate of D3 single cleavage-stage embryo transfer,^{9,17} while an increase in transferred embryos leads to the occurrence of multiple pregnancies.^{11,18} Blastocyst culture requires further screening by prolonging the *in vitro* culture time, which can further eliminate embryos with genetic defects and a high frequency of aneuploidy.^{19,20} In addition, the blastocyst and endometrium can be better synchronized, making single blastocyst transfer a potential effective method to reduce multiple pregnancies while maintaining the clinical pregnancy rate. However, an ideal *in vitro* environment is required for blastocyst culture, which may increase the risk of culture failure and cycle cancellation. Therefore, our center first chose two high-quality D3 cleavage-stage embryos for freezing, and then took D5/D6 blastocysts for secondary freezing. Such a strategy can reduce the risk of cycle cancellation while enabling the advantages of blastocyst selection. Nevertheless, this strategy may also lead to the coexistence of D3 cleavage-stage embryos and D5/D6 blastocysts. Data analysis should be managed to clarify the priority of transfer of D3 cleavage-stage embryos or D5/D6 blastocysts, and this may help formulate a clinical transfer plan.

All of the data included in this study were from FET cycles, and there were no significant differences in the general conditions of the three groups after excluding the interference of non-embryonic factors, such as oocyte quality and endometrial conditions. There was also no significant difference in the survival rate of frozen–thawed embryos among the three groups, which excluded the effect of freezing and thawing operations.

This study showed a significantly higher implantation/thawed rate in the D5-SBT

group compared with that in the D3-DET group, which is consistent with previous studies.^{9,11} Yang et al.⁹ filtered D3 cleavage-stage embryos of high quality by further time-lapse monitoring, but they still found a lower implantation rate compared with that of D5 blastocysts by conventional morphological selection. These results indicate that blastocyst culture is an effective method for selecting embryos with the most developmental potential. We also found that the implantation/thawed rate in the D5-SBT group was higher than that in the D6-SBT group, but the rate of high-quality blastocysts did not vary greatly between these two groups. Our findings are in contrast to those in studies by Sunkara et al.²¹ and Kaye et al.,²² who found that blastocysts on D5 and D6 developed to the same level had similar implantation rates and pregnancy outcomes. However, our results are consistent with those of Sciorio et al.,²³ Desai et al.,²⁴ and Haas et al.²⁵ Taylor et al.²⁶ found that the frequency of aneuploidy of D6 blastocysts was 10% higher relative to that of D5 blastocysts, while D6 blastocysts had a lower implantation rate. These findings might be associated with a delayed development rate (lower embryo development potential), longer *in vitro* culture time, and an increased probability of DNA damage. Moreover, we found that the D6-SBT group had a lower implantation/thawed rate compared with that in the D3-DET group, but this difference was not significant. This finding may be because D3 cleavage-stage embryos had the best morphological score, while D6 blastocysts were obtained after freezing two high-quality embryos. Therefore, the morphological score of D6 blastocysts was lower or equal to that of D3 embryos. The long culture time of D6 blastocysts also indicated relatively low development potential. Interestingly, we found that, in the 11 embryos from the D6-SBT group that

were successfully implanted, only 2 embryos (4BB and 5BB) were of high quality, while the other 9 embryos (7 embryos of 4BC, 1 embryo of 4CB, and 1 embryo of 3BC) did not meet the good-quality standard. This finding suggests that a combination of morphological selection with the days of embryo development may be helpful for better selection of embryos with good development potential. D6 blastocysts that do not meet a good quality should not be easily discarded because they are useful for implantation and final live birth delivery.

Our study showed that the biochemical pregnancy, clinical pregnancy, and ongoing pregnancy rates in the D5-SBT group were not significantly different to those in the D3-DET group. Additionally, the twin pregnancy rate and the risk of preterm birth were lower in the D5-DET group than in the D3-SBT group, which are consistent with previous reports.^{11,27} These findings suggest that transfer of a D5 single blastocyst not only leads to satisfactory pregnancy outcomes, but is also effective in control of the occurrence of multiple pregnancies. Therefore, transfer of a D5 single blastocyst is recommended for transplantation. We also found that the biochemical pregnancy, clinical pregnancy, and ongoing pregnancy rates in the D5-SBT and D3-DET groups were much higher than those in the D6-SBT group. The clinical pregnancy rate in the D5-SBT and D3-DET groups was approximately twice that in the D6-SBT group, while the ongoing pregnancy rate was approximately three times that in the D6-SBT group. These findings indicated that although there was a significantly lower multiple pregnancy rate in the D6-SBT group, the decreased number of transferred embryos and the lower embryo development potential could also result in poor pregnancy outcomes. Additionally, the miscarriage rate in the D6-SBT group was much higher than

that in the other two groups. A chromosome examination of abortion tissue showed abnormalities in all cases of transfer of a D6 single blastocyst, which suggested the potential of a higher proportion of abnormal karyotypes. Taking into consideration the resource waste dependent on recurrent ineffective transplantation, heavy mental, psychological and economic burden on patients, and prolonged pregnancy and live birth time, using D6 blastocysts is recommended as an alternative for final transplantation.

No visible defects, such as major structural malformations, were found in 234 newborns in a follow-up visit. However, there is a probability of an increased risk of epigenetic changes during the blastocyst culture process,⁶ which may cause a skewed sex ratio²⁸ (sex ratio in the D5-SBT group was 1.26:1, while newborns in the D6-SBT group were all girls). Therefore, more attention needs to be paid to the safety of blastocyst transfer, especially the long-term follow-up health data of the offspring.

This was a retrospective study with some limitations. When women had both cleavage embryos and blastocysts available, two D3 cleavage-stage embryos were initially routinely transferred, which resulted in a relatively larger sample size in the D3-DET group than in the D5-SBT and D6-SBT groups. D5/D6 blastocysts were less likely to have a chance to implant compared with D3 cleavage-stage embryos, which was a source of potential bias. Additionally, the comparisons were not paired (i.e., in most cases, the same woman did not undergo double cleavage vs. single blastocyst transfer) to determine the true effect of each transfer. Furthermore, the mean age of the patients included in this study was 31.3 years with good ovarian reserve function. Whether the conclusions of this study are applicable to elderly women still need to be determined

by a large-scale, randomized, controlled study in the future.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and publication of this article: This work was supported by the National Key Research and Development Program of China (No. 2018YFC1004900), the National Natural Science Foundation of China (Nos. 81801544 and 81971350), and the Zhejiang Provincial Department of Education General Research Project (No. Y201840845).

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