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Original Research

Delayed development influences the outcome of different grades of D5 and D6 blastocysts during freeze–thaw cycle

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Abstract: To analyze the effects of blastocysts on the 5th day (D5) and 6th day (D6) of frozen–thawed blastocyst transplantation on pregnancy outcome and provide evidence for further improvement of the strategy. This study included transfers from the Reproductive Medicine Center of the Second Affiliated Hospital of Wenzhou Medical University during freeze–thaw cycles from January 2016 to December 2017. They were divided into D5 group (1616 cases) and D6 group (619 cases) according to blastocyst formation and development. Each group was further divided into 5 groups according to the quality of the blastocyst and the number of transplants, making a total of 10 groups. Following the frozen transplantation cycle, the transplanting rate was significantly higher for D5 (41.73%) than for D6 (23.98%) ($P < 0.05$); the ongoing pregnancy rate (47,40%) was also significantly higher than that of D6 (28.43%) ($P < 0.05$).In the frozen–thawed blastocyst resuscitation transplantation, compared to D6 blastocysts, D5 blastocysts were more conducive to blastocyst implantation and could be used to achieve better clinical pregnancy outcome. In blastocyst selection, a single D5 excellent blastocyst transplant is preferred. Only at the 6th day of non-excellent D6, 2 blastocysts are recommended for transplantation.

Key words: Frozen–thawed blastocyst, Vitrification, Blastocyst transplant, Clinical outcome, Pregnancy rate.

Introduction

With the development of blastocyst culture and freezing technology, blastocyst culture, and vitrification, frozen blastocyst transplantation is increasingly gaining applications in the field of assisted reproductive technology. Many studies have shown that D3 blastocyst transplants are advantageous. In the 2015 American Reproductive Annual Meeting (ASRM) on blastocyst transplant, it was noted that based on the analysis of age, ovarian function, previous treatment history, number of blastocysts, quality etc, transplanting blastocysts in patients with better prognosis has significantly higher live birth rate than by using the same number of cleavage stage blastocysts. However, there is still controversy about the choice of blastocysts and the strategy of transplantation. Some believe that the D5 blastocysts transplanted during the freeze–thaw cycle have a higher clinical pregnancy rate than that by using the D6 blastocysts (1-3). However, some studies have shown that there is no statistically significant difference in pregnancy rates between the two groups (4-6). Because most studies focus on the pregnancy outcome of excellent blastocysts, there are few reports of pregnancy outcomes for non-excellent blastocysts. This study included patients who underwent blastocyst transplantation in the center during the freeze–thaw cycle from January 2016 to December 2017. For this study, we retained patients with low ovarian function, poor blastocyst quality, and economic difficulties. We conducted freezing and transplanting of non-excellent blastocysts to analyze and compare the effects of different grades of D5 and D6 frozen–thawed blastocysts on pregnancy outcomes and the transplantation strategy and value of non-excellent blastocysts.

Materials and Methods

Study subjects

The clinical data of patients with frozen–thawed blastocysts transplanted from the Reproductive Medicine Center of the Second Affiliated Hospital of Wenzhou Medical University from January 2016 to December 2017 were selected. The females were aged 20.4-46.5 years. Exclusion criteria were as follows: endometrium should not be up to 7 mm, after severe intrauterine adhesions. According to the standard, a total of 2278 cycles. All embryos and blastocysts were frozen and resuscitated by vitrification and cryopreservation. After thawing, the blastocysts were cultured according to the time of transplantation.

Freezing and resuscitation of embryo and blastocysts

The standard for freezing blastocysts is to select D5/D6 blastocysts with a rating of 3BC or above. Both embryos and blastocysts were vitrified and resuscitated, the survival criterion of blastocyst resuscitation was the expansion of blastocyst cavity. The blastocyst score was based on Gardner scoring system. Excellent blastocysts were the following: 3AA, 3BB, 3BA, 3AB, 4AA, 4AB, 4BA, 4BB, 5AA, 5AB, 5BA and 5BB and non-excellent

blastocysts were the following: 3BC, 3CB, 4BC, 4CB, 5BC, and 5CB. The thawing of blastocysts was carried out 2 hours before transplantation, and the transplanted blastocysts were frozen and resuscitated before transplantation. The freezing and resuscitating of blastocysts were carried out by the conventional vitrification method of the center (KITAZATO, Japan). *Freezing process:* All cultures were shaken upside down twice, made uniform, preheated to room temperature, and the blastocysts were transplanted to the surface of ES (7.5% dimethyl sulfoxide DMSO + 7.5% ethylene glycol EG) to allow the blastocysts to sink naturally. When the blastocysts were expanded again to more than 80% of their original volume (usually 5-15 minutes), they were transplanted to the frozen solution VS (15% dimethyl sulfoxide DMSO + 15% ethylene glycol EG + 0.5 M sucrose). In the case of repeated absorption, the blastocysts were placed at the forefront of the frozen carrier, the amount of VS was minimized, and 1-2 blastocysts were placed per carrier. The blastocyst-loaded rod was inserted into liquid nitrogen, and the residence time of the blastocyst in VS was generally no more than 1 minute. The handle of the carrier was held in one hand, with a clip held in another hand to clamp the sleeve previously placed in liquid nitrogen, and sthe sleeve was set with the black spot of the load carrier. The setup was put it into the freezing bracket, and stored it in the liquid nitrogen tank. *Thawing process*: The TS was preheated to 37°C, after which the liquid nitrogen was turned on, the front section of the carrier is inserted into the TS at the fastest speed. It was confirmed that the blastocysts had been detached from the carrier and then the carrier is removed under the microscope. The blastocysts were transplanted to DS in about 1 minute, placed in from the surface, and then the liquid slowly spreaded to adapt the blastocyst to DS. In DS for 3 minutes, transplanted to WS1 for 5 minutes, WS2 for 5 minutes, finally transplanted to a pre-prepared culture dish, wash 3 times, and continued the blastocyst to be transplanted. According to the freezing condition of the patient's blastocyst, one or two blastocysts were thawed during the resuscitation cycle, and all blastocysts that were successfully thawed were observed and transplanted after resuscitation.

Endometrial preparation

Hormone replacement cycle: On the third day of menstruation with estradiol valerate (Bulgale, Bayer Pharmaceuticals, Germany) 4-8mg / day, or estradiol tablets 2-4mg / day Fenton, Abbott Biologicals BV Netherlands), adjusted the dose and time according to the endometrial conditions, until the endometrium reached 7-8mm or more. Dydrogesterone tablets (Duffon/Fenton, Abbott Biologicals BV Netherlands) + chenolone 90 mg/day vaginal administration (Chlenone, Merck Serono, Switzerland) were administered. Embryo transfer was performed on day 6 of progesterone administration. Blastocyst transplantation was performed under the guidance of B-ultrasound, and serum β-HCG was monitored until day 14 after transplantation.

Pregnancy outcome evaluation

Clinical pregnancy was assessed 28 days after transplantation by performing B-ultrasound to determine a uterine pregnancy sac, or pathological evidence confirmed by abortion. Clinical pregnancy rate (%): clinical pregnancy cycle/number of transplant cycles × 100. Planting rate (%): number of pregnancy sacs tested by B-ultrasound/number of transplanted blastocysts × 100. Ongoing pregnancy rate (%): pregnancy maintained to more than 12 weeks/number of transplant cycles × 100. Multiple birth rate (%): multiple births/clinical pregnancy × 100.

Statistical analysis

Data were processed using SPSS 18. 0 statistical software. The measured data were expressed as mean ± standard deviation (x ± s), and the mean was compared using a Student's *t*-test. The ratios were compared using Chi square test. Fisher's exact probability method was used, and theoretical frequency was<5. $P < 0.05$ was considered statistically significant.

Results

General information

A total of 2278 frozen–thawed cycles were included in the study, and the general data were retrospectively analyzed. Forty-three blastocysts were eliminated due to degeneration after thawing; hence, the final D5 and D6 transplantation cycles had 1616 and 619 cases respectively (Table 1). There was no significant difference in age, primary/secondary infertility, infertility years, BMI, and endometrial thickness before transformation between the two groups. However, there was a slight difference in the rate of blastocyst resuscitation between the two groups. The D5 group had statistically significant differences in the average number of transplants, blastocyst implantation rate, transplanted excellent blastocyst ratio, clinical pregnancy rate, and sustained pregnancy rate (Table2).

Comparison of pregnancy outcomes of excellent blastocysts at different developmental times

According to the number of blastocysts and the quality of blastocysts, the selected cases were subdivided into 10 groups: D5 group, single (excellent blastocyst) group: 208 cases; single (non-excellent blastocyst) group: 69 cases; double (excellent blastocyst) group: 473 cases; mixed group (one excellent blastocyst and one non-excellent blastocyst): 575 cases; double (non-

Table 1. Basic characteristics of participants in the study.

Group	No.	Age(year)	Primary/Secondary	Infertility (year)	BMI
D5	1616(1641)	31.72+-4.63	674/942 (41.70%/58.30%)	3.66+-2.85	21.664+-3.22
D6	619(637)	31.98+-4.63	270/349 (43.63%/56.37)	3.57+-2.76	21.787+-2.55
P value		0.813	0.413	0.487	0.494

Table2. Comparison of D5 and D6 groups.

Group	No.	Resuscitate transplant rate	Endometrial thickness during transformation	Average transplant number	Blastocyst transplanting rate	Excellent blastocyst transplanting ratio	Clinical pregnancy rate	Ongoing pregnancy rate
D5	1616 (1641)	98.48%	8.98+-1.84	1.82+-0.40	1212/2904 (41.73%)	1729/2955 (58.51%)	893/1616 (55.25%)	766/1616 (47.4%)
D6	619 (637)	97.17%	8.95+-1.89	1.74+-0.44	259/1080 (23.98%)	436/1064 (40.97%)	219/619 (34.89%)	176/619 (28.43%)
P value		0.04	0.869	0.000	0.000	0.000	0.000	0.000

Table 3. Comparison of excellent single blastocyst D5/D6.

Group	n	Age(year)	Clinical pregnancy rate	Ongoing pregnancy rate
SET-D5	208	32.45+-4.76	101/208 (48.55%)	89/208 (42.78%)
SET-D6	91	33.13+-5.39	31/91 (34.06%)	27/91 (29.67%)
P value		0.2628	0.02	0.032

Table 4. Comparison of excellent double blastocyst D5/D6.

Group	n	Age (years)	Clinical pregnancy rate	Ongoing pregnancy rate	Multiple birth rate
DET-D5	473	31.26	315/473(66.59%)	279/473(58.98%)	139/315 (44.12%)
DET-D6	84	31.79	37/84(44.04%)	34/84(40.47%)	11/37 (29.72%)
P value		0.069	0.000	0.002	0.094

Table 5. Comparison of non-excellent single blastocyst D5/D6.

Group	n	Age (years)	Clinical pregnancy rate	Ongoing pregnancy rate
SET-D5	69	32.59+-5.29	22/69(31.88%)	19/69 (27.53%)
SET-D6	87	32.26+-4.64	18/87(20.68%)	15/87 (17.24%)
P value		0.4771	0.140	0.122

Table 6. Comparison of non-excellent double blastocyst D5/D6.

Group	n	Age (year)	Clinical pregnancy rate	Ongoing pregnancy rate	Multiple birth rate
DET-D5	291	31.93+-4.86	132/291 (45.36%)	104/291 (35.73%)	31/132 (23.48%)
DET-D6	180	32.35+-5.57	59/180 (32.77%)	39/180 (21.66%)	6/59 (10.16%)
P value		0.622	0.046	0.000	

Table 7. Comparison of single excellent and non-excellent blastocyst D5/D6.

Group	n	Age(year)	Clinical pregnancy rate	Ongoing pregnancy rate
DET-D5	575	31.93+-4.52	323/575(56.17%)	275/575(48.17%)
DET-D6	177	32.15+-4.22	74/177(41.81%)	63/177(35.56%)
P value		0.531	0.001	0.000

excellent blastocyst) group: 291 cases. D6 group: single (excellent blastocyst) group: 91 cases; single (non-excellent blastocyst): 87 cases; double (excellent blastocyst) group: 84 cases; mixed (one excellent blastocyst and one non-excellent blastocyst): 177 cases; double (non-excellent blastocyst) group: 180 cases.

Excellent single blastocyst D5/D6

We found the clinical pregnancy rate, ongoing pregnancy rate were greater in SET-5 group than in SET-6 group.The difference was statistically significant (Table3).

Excellent double blastocyst D5/D6

We found the clinical pregnancy rate, ongoing pregnancy rate were greater in DET-5 group than in DET-6 group.The difference was statistically significant (Table4).

Comparison of pregnancy outcomes of transplanted non-excellent blastocysts at different developmental times

Non-excellent single blastocyst D5/D6

No differences were noted in the clinical pregnancy rate, ongoing pregnancy rate between SET-D5 group and SET-D6 group (Table5).

Non-excellent double blastocyst D5/D6

The clinical pregnancy rate, ongoing pregnancy rate were greater in DET-5 group than in DET-6 group.The difference was statistically significant (Table6).

Single excellent and single non-excellent blastocyst D5/D6

The clinical pregnancy rate, ongoing pregnancy rate were greater in DET-5 group than in DET-6 group.The difference was statistically significant (Table7).

Discussion

Blastocyst transplant is considered to have a higher clinical pregnancy rate than cleavage stage embryos. This may be because blastocysts are better able to synchronize with the endometrial receptivity and can eliminate some cleavage stage embryos with poor developmental potential or genetic defects (7,8). In the process of frozen blastocyst implantation, age, blastocyst quality, number of blastocyst transplants and endometrial receptivity were associated with pregnancy outcomes. However, infertility years, BMI, and basal FSH were considered to be unrelated to pregnancy outcomes (9).

Whether the pregnancy outcome of D5 blastocysts is better than D6 is currently controversial. Through 15 studies, a meta-analysis of 2,505 transplant cycles Sunkara et al (5) showed higher clinical pregnancy rate [relative risk (RR) 1⁄4 1.14, 95% confidence interval (CI): 1.03–1.26, P 1⁄4 0.01] and considerable higher pregnancy/live birth rate (RR 1⁄4 1.15, 95% CI: 1.01–1.30, P 1⁄4 0.03) with Day 5 compared with Day 6 frozen–thawed blastocyst transplants. Sensitivity analysis of those studies where blastocysts frozen on Day 5 or Day 6 were at the same stage of development showed no significant differences in the clinical pregnancy rate (RR 1⁄4 1.07, 95% CI: 0.87–1.33, P 1⁄4 0.51) and pregnancy/live birth rate (RR 1⁄4 1.08, 95% CI: 0.92–1.27, P 1⁄4 0.36). El-Toukhy (4) et al. also reached a similar conclusion. After analyzing excellent blastocysts, they showed that there was no significant difference between D5 (average number of transplants 1.5+-0.5) and D6 (mean graft number 1.5+-0.5) in clinical pregnancy rate (36% vs 33%), live birth rate (28% vs 28.5%), and abortion rate (41% vs 35%). However, no comparison of non-excellent blastocysts was made. Yang (9) considered that D5 (average number of transplants 1.7+-0.5) was significantly higher than D6 (average number of transplants 1.6+-0.5). After grouping excellent blastocysts and non-excellent blastocysts, there was no significant difference between D5 clinical pregnancy and D6 (52.4% vs 52.6%), and non-excellent blastocysts D5 clinical pregnancy was significantly higher than D6. There was no mention of whether the number of blastocysts transplant after grouping was consistent. In the study of single blastocyst transplant, Ferreux et al. (3) believed that the clinical pregnancy rate and live birth rate of D5 blastocysts were significantly higher than those of D6 blastocysts, both in excellent blastocysts and non-excellent blastocysts. There was no significant difference in abortion rates between the two groups. In this study, there was a significant difference in D5 (mean transplantation 1.82+-0.4) and D6 (mean transplantation 1.74+-0.4) in clinical pregnancy rate (55.25% vs 34.89%) and sustained pregnancy rate (47.4% vs 28.43%). However, because of the statistical difference between the average number of transplants and the rate of excellent blastocysts, after grouping, it was found that in excellent single blastocyst transplant, the clinical pregnancy rate and sustained pregnancy of D5 were higher than D6. In non-excellent single blastocysts, although D5 clinical pregnancy rate, sustained pregnancy rate was higher than D6, but there was no statistical significance and that could be due to less sample size, resulting in no statistical difference. In the double blastocyst transplant (excellent or non-excellent blastocysts), there were statistical differences between the two groups.

Inconsistent results may be related to the age of the enrolled patients, the method of endometrial preparation, quality, number of transplanted blastocysts, blastocyst freezing and thawing protocols, and differences in sample size.Tan Qiao et al (10) analyzed the clinical outcomes of different grades and developmental rate of frozen–thawed blastocysts in women under 35 years of age, and found that although the clinical and continuous pregnancy rates of the D5 group were higher than those of the D6 group, the difference was not statistically significant. Researchers believed that it may have been because the sample number was not large enough. For the preparation of the endometrium, some artificial cycles are used, while in some cases, both artificial and natural cycles are used (9). Although clinical pregnancy rates and continued pregnancy rates are similar for different endometrial preparation protocols based on current literature, some studies have included partial retrospective studies leading to bias and require further RCT studies (11). All the cases enrolled in this study were artificially established to prepare the endometrium with a thickness ≥ 7 mm, which reduced the interference of endometrial receptivity on pregnancy rate. In addition, the blastocyst score is highly subjective and may have an impact on the final result.

It is speculated that the pregnancy rate of D5 is higher than that of D6. The possible reasons are: 1)The lag of developmental time of D6 blastocyst may lead to the change of cell quality. Moreover, even D6 blastocysts of better quality, compared to D5 blastocysts, due to the increased number of blastocysts, resulting in more metabolic activity and larger blastocysts. Still, D5 blastocysts may be more tolerant to freezing and resuscitation injury than D6 blastocysts. These factors may affect the process and outcome of vitrification cryopreservation. (12.13). This study also showed that the overall resuscitation rate of D6 was lower than D5. 2). On the 5th day, the endometrium has better endometrial receptivity to D5 blastocysts (14), but El-Toukhy (4) believed that the implantation window of the endometrium can be extended to the 6th day after fertilization, affecting the blastocysts implantation rate, failure to do so might be a matter of quality in the embryo itself. 3). As the culture time prolonged, the damage of cellular DNA may increase (13). In the PGD/PGS cycle, the detection rate of D5 blastocysts is higher than that of D6, which also increased the implantation rate and clinical pregnancy of D5 frozen blastocysts transplant rate. Leng Qin et al (15) performed aCGH monitoring on D5 and D6 embryos and found that the blastocysts rate of D5 blastocysts was higher than that of D6 transplantation group ($P<0.05$). However, Yang et al. (9) showed no statistical difference in the chromosomal ploidy rate between D5 embryos and D6 embryos by PGS. Alfarawati et al. (16) also showed that aneuploidy was associated with blastocyst formation rate and the quality of blastocyst itself, but not with delayed blastocysts development. Therefore, whether D5 or D6 blastocysts differed in euploid rate required further study.

In this study, D5 blastocysts showed better developmental potential. The transplantation of two excellent D5 blastocysts results in a higher twin rate in ad-

dition to achieving a higher clinical pregnancy rate. In clinical work, selective D5 excellent single blastocyst transplant can enable patients to obtain a higher clinical pregnancy rate without the risk of multiple births. If the patient lacks D5 blastocysts, then transplanting 2 excellent D6 blastocysts can also increase the pregnancy rate (48.55% vs 44.04%) similar to that of a single excellent D5 blastocyst transplant. Nevertheless, the means to avoid the risk of multiple births need to be further explored. For non-excellent blastocysts, whether D5 or D6, the clinical and sustained pregnancy rates of single blastocyst transplants were lower. Transplanting 2 blastocysts can significantly improve the clinical and ongoing pregnancy rates while the double birth pregnancy rate of D5 group is 23.48%, the D6 group is 10.16%. Therefore, it is recommended that if only non-excellent blastocysts are present, especially for D6 non-excellent blastocysts, conducting double transplantation is recommended.

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Competing interests
The authors declare that they have no competing interests

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