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ASSISTED REPRODUCTION TECHNOLOGIES

Pregnancy rates for single embryo transfer (SET) of day 5 and day 6 blastocysts after cryopreservation by vitrification and slow freeze

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Abstract
Purpose The purpose of this study was to compare clinical and ongoing pregnancy rates in cycles with single embryo transfer (SET) of blastocysts cryopreserved on day 5 or day 6. Our aim was to determine whether day 6 blastocysts perform adequately to recommend SET.
Methods Retrospective cohort study including 468 transfer cycles for 392 women younger than age 38 undergoing SET at a university-affiliated IVF clinic in the USA. A total of 261 day 5 blastocysts and 207 day 6 blastocysts for frozen-thawed SET between 2010 and 2016 were analyzed. Data included cryopreservation by both a slow freeze method and vitrification.
Results In total, 59.0% of day 5 SET cycles resulted in a clinical pregnancy compared to 54.1% of day 6 blastocysts (p = 0.54). Ongoing pregnancy rates from day 5 frozen-thawed blastocysts (51.7%) were comparable to day 6 (44.9%, p = 0.14). When looking at vitrified blastocysts only, there were no significant differences between day 5 and day 6 blastocysts, with a clinical pregnancy rate of 69.2% for day 5 and 72.5% for day 6 (p = 0.68).
Conclusions SETs of day 6 cryopreserved blastocysts resulted in similar clinical and ongoing pregnancy rates compared to day 5, particularly after vitrification.

Keywords Single embryo transfer · Day 5 blastocyst transfer · Day 6 blastocyst transfer · Frozen embryo transfer · Delayed blastulation

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Introduction

Elective single embryo transfer (SET) has become a mainstay in meeting the goals of IVF: a healthy, full-term, singleton pregnancy and reduction of multiple gestations [1]. It is imperative to consider all embryo selection tools that allow centers to perform successful SET while minimizing a decrease in pregnancy rates. Single embryo transfer remains the most effective way to decrease the rate of multiple gestation and avoid the significant consequences of maternal and neonatal morbidity. As there is evidence that single blastocyst transfer does not result in decreased pregnancy rates or live birth rates [2–6], even when performed with cryopreserved embryos, there must be an effort to utilize this strategy in all feasible situations.

Effective SET relies on the ability to select the embryo most likely to thrive, and extended culture to the blastocyst stage facilitates this selection with the assumption that genetically or developmentally impaired embryos are less likely to survive to the blastocyst stage. Fresh single blastocyst transfers result in excellent pregnancy rates [2, 7]. However, some embryos have reached blastulation by day 5 and others not until day 6 or even day 7, raising the question: Are blastocysts with a delayed rate of blastulation suitable for SET while maintaining acceptable pregnancy rates?

A comparison of day 5 and day 6 embryos has been performed in a number of prior studies. In a fresh blastocyst transfer cycle, day 6 embryos are outperformed by day 5 embryos, largely attributed to endometrial asynchrony [8]. Therefore, most available studies explore the thaw and transfer of these cryopreserved blastocysts in a subsequent cycle. Some studies suggest that day 6 blastocysts have equivalent clinical outcomes compared to day 5 blastocysts in frozen-thawed cycles [8–16], while other studies, including a review from 2010, suggest clinical outcomes are better for day 5 blastocysts [17–20]. More recently, Desai et al. found significantly higher

implantation rates and live birth rates in day 5 blastocyst transfers compared to day 6 when evaluating 354 frozen embryo transfers [21]. While a number of these studies have attempted to evaluate pregnancy rates of a single embryo by reporting implantation rates, none of the abovementioned studies looked specifically and exclusively at SET cycles in the setting of delayed blastulation. Haas et al. evaluated a subset of transfers that were specifically SET and found day 5 clinical pregnancy rates (41.9%) were significantly higher than day 6 (22.2%) [22]. Kang et al. compared clinical outcomes between single and double frozen-thawed blastocyst transfer of either day 5 or 6 embryos, exhibiting significantly lower clinical and ongoing pregnancy rates following day 6 SET compared to day 6 double blastocyst transfer [23]. However, they did not perform a direct comparison between day 5 and day 6 SETs. The question remains whether blastocysts cryopreserved on day 5 have an advantage over blastocysts with delayed development that do not reach this stage until day 6, and whether day 6 SET should be encouraged. Thus, the aim of the present study was to determine whether clinical and ongoing pregnancy rates for SET of day 6 cryopreserved blastocysts are equivalent to day 5 SET.

Materials and methods

We performed a retrospective cohort analysis of 468 transfer cycles from 392 women from February 2010 to March 2016. Women of all diagnoses were under age 38 at the time of oocyte retrieval and underwent SET of frozen-thawed autologous blastocysts cryopreserved on day 5 or day 6. Patients opting for preimplantation genetic screening were excluded. We recorded baseline (age at oocyte retrieval and transfer, markers of ovarian reserve, gravidity, parity, diagnoses, BMI, and previous cycle outcomes) and cycle characteristics (including fresh cycle serum levels, follicles measured, oocytes retrieved, method of fertilization, cycle outcome, number of embryos cryopreserved, cryopreservation method, and frozen cycle data such as endometrial preparation method, elective versus obligatory SET, and reproductive outcomes). Clinical outcomes included the primary outcome of clinical pregnancy (ultrasound confirmation of intrauterine gestational sac), as well as ongoing pregnancy (pregnancy progressing beyond 12 weeks gestational age). Data were attained from our electronic medical record. Live birth data were not available for five ongoing pregnancies at the time of analysis. Study approval was obtained by the University Institutional Review Board.

Pregnancy outcomes were collected for cycles when available (n = 360) to assess obstetric and neonatal complications including NICU admissions, congenital anomalies, preterm delivery, preeclampsia, and gestational diabetes. This information was gathered from verbal or written accounts directly from patients.

Patients underwent controlled ovarian stimulation as previously described [24]. Vaginal oocyte retrieval was performed 35 h after trigger. Oocytes were fertilized using either ICSI (87.9%) or conventional insemination with a fertilization check performed 16–18 h post-insemination or ICSI. Most embryos were cultured to the blastocyst stage in sequential media (SAGE® Quinn's Advantage Cleavage Medium and Blastocyst Medium), except 12.8% cultured in single-step media (LifeGlobal®) at the end of the study period. Good quality blastocysts available after fresh embryo transfer on day 5 (n = 441), or as a result of a "freeze all" cycle (n = 27), were cryopreserved. Reasons to freeze all embryos included elevated progesterone ≥1.5 ng/mL, increased risk of OHSS, or evidence of uterine pathology such as a polyp warranting surgery before transfer. Those blastocysts not reaching the expanded stage by day 5 remained in culture and, if considered good quality on day 6, were cryopreserved. Blastocysts were graded according to degree of expansion, quality of the inner cell mass, and quality of the trophectoderm cells using the Gardner scoring system [25]. Blastocysts with a Gardner score of 3BB or better were considered good quality and suitable for cryopreservation.

A slow freezing protocol using gradual exposure from 5% glycerol to 9% glycerol and 0.2 M sucrose was used prior to January 2013, and embryos were frozen in vials. After January 2013, vitrification with rapid exposure to a cryoprotectant solution of 15% ethylene glycol, 15% DMSO, 20% dextran, and 0.5 M sucrose was used for all blastocyst cryopreservation in a Cryolock® device (Irvine Scientific). Blastocoeles were collapsed using a laser pulse of 300 μs (constant 0.9 joules) applied to the junction of two trophectoderm cells prior to vitrification only.

All blastocysts were rapidly warmed into solutions of 0.5 and 0.2 M sucrose and rinsed through HEPES-buffered human tubal fluid with 12 mg/ml human serum albumin. Embryos were thawed 1–2 h before transfer and allowed to incubate for re-expansion. Embryos were equilibrated in culture 1–2 h before transfer.

Transfers in a programmed or natural cycle (depending on whether the patient was ovulatory) were performed 6 days after the luteinizing hormone surge or on the sixth day of progesterone (P) administration respectively. Endometrial preparation for programmed cycles utilized leuprolide acetate for pituitary suppression with stepwise estradiol (E2) patches and luteal phase intramuscular P injections, with monitoring of serum E2 and P levels as well as an ultrasound to evaluate the endometrial thickness prior to transfer as described elsewhere [26]. Natural cycles were monitored with serum-luteinizing hormone to determine the day of surge, and transfer was performed 6 days later. Patients received vaginal P support in the luteal phase with Crinone® 90 mg/day or Endometrin® 100 mg twice daily.

Data was analyzed using the Statistical Package for the Social Sciences (Release 24.0, SPSS, Inc.), and results are

presented as mean ± SD unless otherwise stated. Independent *t* test, Pearson's chi-square test, and Fisher's exact test were used for continuous or categorical variables respectively. A binomial logistic regression was performed evaluating clinical pregnancy after day 5 or day 6 frozen-thawed SET controlling for covariates including age at time of retrieval, number of oocytes retrieved in fresh cycle, live birth from fresh cycle, "freeze all" fresh cycle, endometrial preparation method, cryopreservation method, ICSI versus conventional insemination, and sequential versus global media culture. Differences were considered statistically significant with a p value <0.05. We performed a post hoc sample size calculation based on approximate sample sizes of 225 per group; a two-group continuity corrected chi-square test with a two-sided α of 0.05 would have 80% power to detect a difference between clinical pregnancy rates of 47 versus 60%, comparable to our calculated pregnancy rates.

Results

Table 1 describes the total number of SET cycles at each blastocyst age and the patient characteristics of each group. There were no notable differences between the day 5 and day 6 groups with respect to primary diagnosis, not listed in the table. There were more embryos cryopreserved by the slow freezing method than by vitrification due to the standard practices for our lab and recent adoption of vitrification during the inclusion time frame. Fewer than 10% of transfers were performed after a "freeze all" stimulation cycle. There were more elective SET performed in the day 5 group and more obligatory SET performed in the day 6 group, reflecting our preference for embryos of equivalent grading with a faster rate of blastulation. Though the thaw survival rate was not evaluated in this study, our center during this time had a thaw survival rate of about 80% after slow freeze and >98% after vitrification. In total, 16% of the women included in this study underwent up to three cycles (n = 62; 52 women with two cycles, 10 women with three cycles).

The reproductive outcomes by SET day 5 versus day 6 (Table 2) revealed no differences with respect to clinical pregnancy rate, ongoing pregnancy rate, live birth rate, miscarriage rate, or rate of multiple gestations (clinical pregnancy rate unadjusted OR 1.2 [95% CI 0.85–1.76]). Clinical pregnancy rates in patients under age 35 were 60.5% for day 5 and 58.1% for day 6 blastocysts. This is consistent with our center's fresh day 5 SET during this time period (clinical pregnancy rate 63%). The multiple gestation rate was less than 2% in each group due to monozygosity; two twin sets resulted from programmed cycles and one from a natural cycle. Live birth data was available for all cycles apart from seven ongoing pregnancies (four from day 5 versus three from day 6), where information regarding delivery was not yet available at the time of writing the manuscript. There were no differences between day 5 and day 6 blastocyst transfers in each age group, nor were there differences between clinical or ongoing pregnancy rates when comparing younger and older women. Women undergoing elective SET had a 62.4% clinical pregnancy rate with day 5 blastocysts compared to 59.8% with day 6 blastocysts (p = 0.69).

Table 1 Characteristics of single embryo transfer cycles by day of blastocyst cryopreservation

	Day 5 SET	Day 6 SET	p value
Age at time of cryopreservation, years	32.11 ± 2.88	32.42 ± 2.89	0.26
Age at time of ET, years	33.49 ± 3.22	34.01 ± 3.14	0.08
BMI, kg/m^2	26.31 ± 6.05	26.18 ± 5.96	0.82
AMH, ng/mL	5.23 ± 6.71	4.03 ± 3.91	0.07
Multiparous, n (%)	168 (64.4)	132 (63.8)	0.92
Total frozen ET cycles, n	261	207	0.08
<35 years old	205 (78.5)	148 (71.5)	
35–37 years old	56 (21.5)	59 (28.5)	
Endometrial preparation, n (%)			0.98
Natural with luteal progesterone	57 (21.8)	45 (21.7)	
Programmed	204 (78.2)	162 (78.3)	
Cryopreservation technique, n (%)			0.02
Slow freeze	170 (65.1)	156 (75.4)	
Vitrification	91 (34.9)	51 (24.6)	
Cycles following "Freeze all" n (%)	22 (8.4)	5 (2.4)	0.01
Elective or obligatory SET n (%)			
Elective	186 (71.3)	82 (39.6)	<0.01
Obligatory	75 (28.7)	125 (60.4)	

Table 2 Reproductive outcomes of SET cycles, overall, and by age group

	Day 5 SET	Day 6 SET	*p* value
Clinical pregnancy, *n* (%)	154/261 (59.0)	112/207 (54.1)	0.54
<35 years old	124/205 (60.5)	86/148 (58.1)	0.68
35–37 years old	30/55 (54.5)	27/59 (45.8)	0.35
Ongoing pregnancy/live birth, *n* (%)	135/261 (51.7)	93/207 (44.9)	0.14
<35 years old	112/205 (54.6)	72/148 (48.6)	0.27
35–37 years old	23/56 (41.1)	21/59 (35.6)	0.55
Multiple gestation, *n* (%)	2/154 (1.3)	1/112 (0.9)	1.00
Clinical pregnancy loss, *n* (%)	22/154 (14.3)	20/112 (17.9)	0.54
Biochemical pregnancy, *n* (%)	28/184 (15.2)	25/138 (18.1)	0.51
Ectopic pregnancy, *n* (%)	1/184 (0.5)	0/138 (0)	1.00

When analyzing first cycles only to account for couples with multiple cycles within our inclusion timeframe, the clinical pregnancy rate was 62.1 versus 55.2% for day 5 versus day 6 ($p = 0.18$). For vitrified first cycle SETs only, the results were comparable to our overall findings with a clinical pregnancy rate of 69.1 versus 71.1% ($p = 0.84$).

Upon adjusting for potential confounders, logistic regression generated an adjusted odds ratio for clinical pregnancy of 1.16 ([95% CI 0.79–1.72], $p = 0.44$) when comparing day 5 to day 6 SET, confirming no difference between the groups. The analysis determined that cryopreservation method and use of global media were predictive of clinical pregnancy ($p < 0.05$), but age at time of retrieval, number of oocytes retrieved, natural versus programmed frozen-thawed transfer, use of ICSI, live birth from the fresh cycle, and embryos from a previous "freeze all" cycle were not significantly predictive. These findings prompted the stratified analysis by cryopreservation method (Table 3).

As technology has changed and vitrification has become the preferred method for blastocyst cryopreservation, we have evaluated the blastocysts separately based on freezing method. In the slow frozen group, day 5 and day 6 blastocysts did not have different clinical and ongoing pregnancy rates. There was a difference in the performance of day 6 blastocysts that were cryopreserved by vitrification versus slow freeze, resulting in an ongoing pregnancy rate that increased from 39.1 to 62.7% ($p = 0.004$). While overall reproductive outcomes were higher in the vitrification group compared to slow freeze, again there were no differences between day 5 and 6 SETs within cryopreservation method.

No differences were observed in neonatal or obstetric outcomes. There were 15 neonatal complications in the day 5 group versus 8 in the day 6 group (11.7 versus 9.0% of live births, respectively) and 17 major or minor neonatal anomalies (7 from day 5 versus 10 from day 6). Sixteen cycles (9 versus 7) required NICU admission for reasons such as prematurity, meconium aspiration, or neonatal surgery. There were two cases of neonatal death, both from day 6 blastocysts, one after delivery at 25 weeks due to HELLP syndrome, and one after complications of undiagnosed vasa previa requiring emergent cesarean section and resuscitation.

Obstetric complications included primarily diabetic disorders (23 cases, 14 versus 9) and hypertensive disorders

Table 3 SET for slow frozen and vitrified cryopreserved embryos, overall, and by age group

	Slow frozen			Vitrified		
	Day 5	Day 6	*p* value	Day 5	Day 6	*p* value
Total frozen ETs, *n*	170	156		91	51	
Clinical pregnancy, *n* (%)	91/170 (53.5)	75/156 (48.1)	0.55	63/91 (69.2)	37/51 (72.5)	0.68
<35 years old	70/128 (54.7)	59/114 (51.8)	0.57	54/77 (70.1)	27/34 (79.4)	0.31
35–37 years old	21/42 (50.0)	16/42 (38.1)	0.36	9/14 (64.3)	10/17 (58.8)	0.76
Ongoing pregnancy/live birth, *n* (%)	78/170 (45.9)	61/156 (39.1)	0.22	57/91 (62.6)	32/51 (62.7)	0.99
<35 years old	61/128 (47.7)	49/114 (43.0)	0.47	51/77 (66.2)	23/34 (67.6)	0.88
35–37 years old	17/42 (40.5)	12/42 (28.6)	0.25	6/14 (42.9)	9/17 (52.9)	0.58
Multiple gestation, *n* (%)	1/91 (1.1)	0/75 (0)	1.00	1/63 (1.6)	1/37 (2.7)	1.00
Clinical pregnancy loss, *n* (%)	15/91 (16.5)	15/75 (20.0)	0.81	7/63 (11.1)	6/37 (16.2)	0.55
Biochemical pregnancy, *n* (%)	16/107 (15.0)	23/100 (23.0)	0.14	12/77 (15.6)	1/38 (2.6)	0.06
Ectopic pregnancy, *n* (%)	0 (0)	0 (0)		1/77 (1.3)	0 (0)	1.00

including preeclampsia (21 cases, 12 versus 9). There were eight cases of abnormal placentation (0.7% in day 5 vs. 8.0% in day 6, $p < 0.05$) including five cases of placenta previa.

Discussion

As SET is becoming widely accepted to promote healthy singleton pregnancies after IVF, our findings further support this recommendation for blastocysts cryopreserved on day 6 as well as day 5. The rate of ongoing pregnancy is equivalent, allowing us to confidently recommend SET for our young patients independent of rate of blastulation. In turn, this study justifies adoption of vitrification and day 6 blastocyst SET to decrease the multiple gestation rate after assisted reproductive technology without an appreciable decrease in pregnancy outcomes.

Most notably, high clinical pregnancy rates were achieved using vitrified blastocysts, regardless of the day of cryopreservation, with a clinical pregnancy rate around 70%, even when assessing a slightly older (35–37 years) sample. While these numbers are small, they represent the way in which vitrification has leveled the playing field for SET, and our study demonstrates that this extends to blastocysts with delayed blastulation.

Similar studies have reviewed various aspects of blastocyst transfer and SET, but to our knowledge, this is one of few studies comparing day 5 and day 6 cryopreserved blastocyst SETs exclusively. Haas et al. retrospectively evaluated 791 freeze-thaw cycles, and within this, a subset of transfers was specifically SET, with 203 day 5 and 157 day 6, similar in size to our study [22]. Unlike the present study, they found a significant decrease in day 6 clinical pregnancy rates (41.9 vs. 22.2% in day 5 vs. day 6) [22]. However, these findings were within the context of a larger study with clinical and ongoing pregnancy rates from 40 to 50% with multiple embryo transfer, and their pregnancy rates generally appear to differ from those reported in our study. It could be argued that prior studies reporting implantation rates in addition to clinical pregnancy rates could provide information to extrapolate into the setting of SET. Reviewing numbers from the studies cited, the implantation rates for day 5 blastocysts range from 16 to 50% compared to 16–41.5% for day 6. As these implantation rates are substantially lower than the pregnancy rates we report, there is evidence that implantation rates may not be an accurate estimation of SET pregnancy rates, nor do they further solidify the evidence justifying elective SET as the best means to reduce multiple gestations while maintaining acceptable pregnancy rates.

As a retrospective study, our findings are limited in applicability as they include cryopreserved blastocysts by a slow freeze method in addition to vitrification which has now become standard practice. The 72.5% clinical pregnancy rate and 62.7% ongoing pregnancy/live birth rate in vitrified day 6 blastocysts are markedly improved over the rates of slow freeze embryos, owing to the advances in technology, but it should be noted that there were fewer vitrification SET cycles due its recent introduction in our lab. While transfers after vitrification would certainly lend more current information, we feel that outcomes from slow frozen blastocysts are worthy of review as many patients have slow frozen embryos in storage. The generalizability of this study must be considered as we excluded older women due to the age-based recommendations from the American Society for Reproductive Medicine for fresh embryo transfers [27]. Finally, we opted to review cycles rather than patients; a common concern is that multiple cycles in the same patient should not be analyzed independently. In our study, 15.8% of patients had more than one included transfer cycle, and we have provided results when including only the first cycle from each couple for comparison.

While there was no statistically significant difference in slow freeze blastocysts on day 6, there may be a clinically notable difference when compared to the performance of day 5 slow frozen blastocysts. Another possible confounder affecting our slow freeze results was the hesitance in the past to culture blastocysts to full expansion and a bias toward day 5 cryopreservation due to the increased fluid in the blastocoele and the risk of damage due to increased ice crystal formation. At the time of slow freeze, cryopreserved blastocysts from day 6 may have seen increased structural damage, and as a result, their performance may have suffered. It should be noted that our study suggests that vitrification has eradicated this risk, further promoting the practice of culturing until full expansion such that morphology, and trophectoderm morphology, in particular, can be used for predictive embryo selection [28].

Women ages 35–37 had lower pregnancy rates than those younger than 35 years, particularly when receiving a day 6 blastocyst (ongoing pregnancy rate 35.6 vs 48.6%, $p = 0.09$). While not statistically different, there may be clinical implications. In this subset of women, elective SET of a day 6 frozen blastocyst may not be a reasonable option.

All frozen embryos considered for transfer in this study were of good quality despite delayed blastulation in the day 6 cryopreservation group, and therefore, our decision to continue to recommend SET is based on previous literature. Previous studies suggest morphologic grading, and evaluation at the time of vitrification and post-thaw may be more important than time passed in vitro and rate of development [19, 28–32]. Furthermore, hatching status may be even more indicative of clinical outcome than grading [32]. When available, the use of preimplantation genetic screening has been added to the assessment and selection of the embryo. Prior studies have found no difference in aneuploidy rates between day 5 and day 6 embryos [15, 31, 33, 34], and while this modality has been used to minimize multiple births, it remains

unclear whether this modality is necessary in this patient population to optimize pregnancy outcomes.

Day 6 vitrified blastocysts performed similarly to day 5 blastocysts and were equally suitable for SET in the right patient population. SET for decreasing multiple gestation rates ought to be recommended.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study, formal consent is not required.

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