Published in final edited form as:

Fertil Steril. 2008 December ; 90(6): 2138–2143. doi:10.1016/j.fertnstert.2007.10.029.

Blastocyst development rate impacts outcome in cryopreserved blastocyst transfer cycles

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Structured Abstract

Objective—To assess cycle outcome among day 5 and day 6 cryopreserved frozen-thawed blastocyst embryo transfers (FBET).

Design—Retrospective cohort study.

Setting—Military-based ART center.

Patients—One hundred seventy-two non-donor, programmed cryopreserved embryo cycles.

Interventions—Fully expanded blastocysts on day 5 were cryopreserved on day 5 and those achieving this state on day 6 were cryopreserved on day 6. Leuprolide acetate was given for ovulation inhibition and endometrial supplementation was by oral and vaginal estradiol. Progesterone in oil was administered and blastocyst transfer occurred in the morning of the 6th day of progesterone.

Main Outcome Measures—Implantation, pregnancy and live birth rates.

Results—Fresh and frozen cycle characteristics were similar between groups. Day 5 FBET had significantly higher implantation rates (32.2% vs. 19.2%; $p=0.01$) which remained significant even

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Where the work was done: Walter Reed Army Medical Center ART Program, Washington, DC and the Reproductive Biology and Medicine Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD

Conflict of interest: None

Disclosure: The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of Health and Human Services, the Department of the Army or the Department of Defense.

Presented at a meeting: Presented in part at the 62nd annual meeting of the American Society for Reproductive Medicine, New Orleans, LA, October 21-25, 2006

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when adjusting for co-variables (OR: 1.91; 95%CI: 1.00, 3.67). Live birth rates trended towards improvement after adjusting for co-variables (OR: 1.18; 95%CI: 0.61, 2.30).

Conclusions—Cryopreserved day 5 blastocysts have higher implantation rates and trend toward improved pregnancy outcomes compared to cryopreserved day 6 blastocysts. This suggests that embryo development rate may, in part, predict implantation and subsequent FBET outcomes although embryos not achieving the blastocyst stage until day 6 still demonstrate acceptable outcomes.

Keywords

frozen-thawed blastocyst cycle; embryo transfer; day 5 vs. day 6

Introduction

An advanced understanding of the metabolic requirements and developmental needs of *in vitro* embryos has led to enhanced culture media and incubation systems. Presently, greater than 50% of all zygotes produced develop into blastocysts (1,2). Extended culture time allows for the assessment of embryo physiology by examining morphology beyond genomic activation; a reflection of embryonic potential as opposed to innate oocyte processes (3). This prolonged assessment allows for the selection of the most suitable embryos to transfer with resultant improvements in implantation rates (IR) (4).

Many factors influence the likelihood of blastocyst development and transfer: female patient age and the number of oocytes retrieved, for example (2). Up to two-thirds of embryos develop into blastocysts by day 5 in fresh-cycle assisted reproduction technology (ART) (4) and those achieving the expanded blastocysts stage have outstanding IR approaching 70% in some studies (5). When examining fresh ART cycles with blastocysts remaining after transfer, those with “spare” blastocysts demonstrated significantly higher IR (44.8% vs. 26.7%; $p=0.024$) and pregnancy rates (PR) per transfer (72.4% vs. 44.4%; $p=0.012$) compared to those without blastocysts remaining (6). Not only are fresh cycle outcomes improved, but also having remaining blastocysts permits blastocyst cryopreservation and subsequent frozen blastocyst embryo transfers (FBET).

While reports examining fresh cycle blastocyst transfer outcomes appear consistent, investigations of FBET cycle outcome are limited and the results are less clear. One study suggested that blastocysts frozen on day 5 or 6 have higher PR compared to those frozen on day 7 or 8 (38.9% vs. 6.2%; $p=0.04$) (7). However, subsequent studies have failed to corroborate this finding (8,9).

When examining both cryopreserved autologous and cryopreserved oocyte donation FBET cycles, no differences in IR or clinical PR were observed among day 5 blastocysts compared to day 6 blastocysts (10). However, day 7 cryopreserved blastocysts demonstrated a significantly reduced clinical PR (14.6% vs. 28.0%; $p=0.04$) and a trend towards reduced IR, suggesting that developmental rate may be an important prognostic marker for cycle outcome.

Given the conflicting results of studies suggesting that the development rate of blastocysts influences cycle outcome, we set out to examine the transfer cycle outcome of autologous blastocysts cryopreserved on day 5 to those with slower development that were cryopreserved at the blastocyst stage on day 6.

Materials and Methods

Study type and analysis

After obtaining Walter Reed Army Medical Center Institutional Review Board approval, a retrospective analysis from January 2000 to December 2005 was performed. Included in the analysis were non-donor, programmed cycles undergoing transfer of blastocyst- embryos cryopreserved on either culture day 5 or 6. Only the first FBET after the initial fresh ART cycle was analyzed and no donor oocyte cycles were performed. Embryos progressing to the blastocyst stage on day 5 were cryopreserved on day 5 and classified as day 5 blastocysts. Those reaching satisfactory morphology on day 6 were cryopreserved on day 6 and classified as day 6 blastocysts. There were no embryos cryopreserved after day 6. Transfer cycles that had a combination of day 5 and day 6 cryopreserved blastocysts were excluded. Data collected included age at cryopreservation and at cryopreserved blastocyst transfer, day 3 FSH preceding cryopreservation, number of oocytes retrieved and number of embryos cryopreserved, thawed, survived, and transferred. Transfer difficulty and embryo quality data were also evaluated.

Outcome data included implantation, spontaneous abortion, biochemical pregnancy, clinical pregnancy and live birth. Biochemical pregnancies were not considered as implantations or as pregnancies. At 6-7 weeks gestation a transvaginal ultrasound was performed to determine sac number and viability. For purposes of the analysis, clinical pregnancies were determined by ultrasound and excluded spontaneous abortions. IR was the number of gestational sacs per number of embryos transferred. Cycle characteristics and IR were assessed using Student's t-tests and chi-square; generalized estimating equations (GEE) were used for multivariable models (SAS 9.1; SAS Institute, Cary, NC).

Fresh cycle stimulation protocol

Fresh-cycle ovarian hyperstimulation was accomplished using a combination of long luteal gonadotropin-releasing hormone (GnRH) agonist (leuprolide acetate (LA); TAP Pharmaceuticals, Deerfield, IL) or micro-dose flare GnRH agonist (LA, 40 µg twice daily) based on the initial clinical evaluation. For either stimulation protocol, patients received a combination of recombinant FSH (Gonal-F; EMD Serono, Rockland, MA) and human Menopausal Gonadotropins (Repronex; Ferring Pharmaceuticals, Suffern, NY) twice daily and dosages were adjusted based on the anticipated follicular response. Follicular development was monitored by transvaginal ultrasonography. Once at least 4 follicles reached ≥ 16 mm, 10,000 IU hCG was administered. Oocyte retrieval was performed 34 to 36 hours later.

Embryo culture protocol

Fertilization was performed 3 to 6 hours post-retrieval by either conventional insemination in HTF medium supplemented with 5% HSA (InVitro Care, Frederick, MD) or by intracytoplasmic sperm injection (ICSI) as indicated (Day 0). Oocytes inseminated by ICSI were cultured overnight in either supplemented HTF medium (prior to July 2004) or in supplemented Global[®] Medium (Life Global, Canada) (after July 2004). Evidence of fertilization was determined at approximately 18 hours post-insemination by the presence of two pronuclei.

All fertilized zygotes were placed into either sequential P-1[®] medium (Irvine Scientific, Santa Ana, CA) and CCM medium (Vitrolife, Englewood, CO) supplemented with 5% HSA or Global[®] Medium used in a non-sequential manner. This medium was supplemented with 10% HSA and also used for microdroplet culture, under oil, in 5% CO₂, for days 1 through 6. Fresh media was made and used on day 3 to refresh the culture conditions for all embryos to be placed into extended culture conditions.

Patients having >5 high-grade embryos on day 3 of embryo development were candidates for blastocyst transfer. On day 5 or day 6, those embryos achieving the blastocyst stage were evaluated morphologically and the two best appearing were selected for transfer. For those patients not meeting these criteria, day 3 embryo transfers were performed and the remaining embryos were placed in extended culture media. A modified grading system (11) was used to determine the developmental level and to score both the inner cell mass (ICM) and the trophectoderm (TE) as follows: A) cell number is high and there is good cellular adhesion; B) cell number is lower with inferior cellular adhesion; and C) no apparent ICM and the TE has few or highly granulated cells. Any blastocyst earning a score of B/B (ICM/TE) or better was cryopreserved.

Cryopreservation protocol

Remaining blastocysts were cryopreserved on day 5 or 6 using Menezo's two-step slow-freezing method (12). The blastocysts were exposed for 10 minutes in 5% glycerol + base medium (Hepes-buffered HTF with 10% HSA; In Vitro Care, Frederick, MD; Sage, Trumbull, CT) at room temperature (25°C), then moved to 9% glycerol + 0.2M sucrose. They were then loaded into straws (IMV Technologies, L'Aigle, France), sealed and placed into a rate-controlled freezer (CL-8000, Biogenics, Napa, CA). The initial blastocyst cooling was at a rate of 1°C/min to -7°C, starting at 18°C. Using a cold, non-sterile cotton-tipped applicator, ice crystallization was manually induced by touching the straw. The -7°C temperature was held for 10 minutes. Further cooling continued at a rate of 0.3°C/min to -35°C, at which time the straws were directly plunged into liquid nitrogen (-196°C) for cryostorage.

Programmed FBET protocol

As part of the programmed cycle, daily 0.3 mg norgestrel/0.03 mg ethinyl estradiol tablets (Lo-ovral; Wyeth Pharmaceuticals, Collegeville, PA) began on day 2/3 of a natural menstrual cycle. On cycle day 14, leuprolide acetate (Lupron, 1 mg/day) was then started followed by a serum estradiol level on cycle day 3 of the following cycle. Once below 50 pg/mL, endometrial supplementation with increasing dosages (2 mg-6 mg) oral micronized estradiol (Estrace; Warner Chilcott, Rockaway, NJ). If serum estradiol concentrations were <300 pg/mL, 2 mg of micronized estradiol was administered vaginally every night. Once serum estradiol concentrations reached ≥ 300 pg/mL and an ultrasonic endometrial stripe thickness of ≥ 7mm with a proliferative pattern, daily progesterone in oil (50 mg daily) was administered intramuscularly and continued through 8 weeks gestation. Doxycycline began concurrently with the progesterone and was discontinued the evening prior to transfer. FBET occurred on the morning of day 6 of progesterone support.

Blastocyst transfer Technique

A mock embryo transfer and saline sonohysterogram were performed in all patients prior to enrollment. Blastocysts were thawed by removing the straw from the liquid nitrogen and held at room temperature for approximately 30 seconds. The straw was opened and the contents were expelled into a 60 mm petri dish. Once identified, the blastocysts were re-hydrated in a series of 7 cryoprotectant dilutions at room temperature for 5 minutes each. The blastocysts were then washed in the base medium and subsequently ready for transfer. All FBET were performed with a full bladder under direct ultrasound guidance (Ultramark 4 with a 5 MHz transducer; ATL, Bothell, WA) using the Wallace embryo transfer catheter (Smiths-Portex, Kent, UK) via the afterload technique previously described (13).

Results

Day 5 cryopreserved blastocysts were transferred in 100 (58.1%) patients and 72 (41.9%) received day 6 cryopreserved blastocysts. Thirteen additional FBET cycles were excluded from

analysis because a combination of day 5 and day 6 cryopreserved blastocysts were transferred. Supranumerary embryos were the indication for cryopreservation in 97.1% of the study population; 5 patients had embryos frozen due to the inability to perform the fresh cycle transfer. During the study interval, one ectopic pregnancy occurred in the day 5 FBET group and was excluded from analysis.

Table 1 shows patient characteristics during the fresh ART cycle generating the cryopreserved blastocysts. The mean patient age, fresh cycle day 3 FSH, and number of embryos cryopreserved did not differ between groups. During the study interval our program transitioned from sequential embryo culture media to Global[®] Medium non-sequential media. There were no differences between groups with regard to media used; sequential media usage accounted for the vast majority of cycles among both groups during the study interval (day 5: 96.0% vs. day 6: 95.8%; $p=0.96$). The mean number of blastocysts cryopreserved was 3.8 ± 2.3 for the day 5 group and 3.5 ± 2.0 for the day 6 group ($p=0.45$). Those cycles generating day 5 cryopreserved blastocysts demonstrated a higher fresh cycle clinical PR than those generating day 6 blastocysts for cryopreservation (53.6% vs. 43.7%); however this did not reach statistical significance ($p=0.21$). A higher proportion of those cycles in which blastocysts were cryopreserved on day 6 had undergone a fresh day 3 transfer compared to blastocysts cryopreserved on day 5 (40.8% vs. 33.3%; $p=0.30$), though this difference was not statistically significant.

Cycle characteristics and pregnancy outcomes were analyzed and are reported in Table 2. No significant differences in patient age at the time of cryopreserved blastocyst transfer were observed between groups. In the cohort, the cryopreserved blastocyst survival rate was greater than 92%; there were no differences between groups. Furthermore, the mean number of blastocysts transferred was 2.2 and did not differ between groups. There were no overall differences in blastocyst morphology between groups. Ultimately, day 5 FBET yielded significantly higher IR (32.2% vs. 19.2%; $p=0.01$). While there was a 42% relative increase in clinical PR among the day 5 FBET group, this was not statistically significant ($p=0.33$). Because there were no late pregnancy losses, the ongoing PR was the same as the live birth rate (LBR).

The unadjusted odds for implantation between day 5 and day 6 groups was 1.96 (95% CI: 1.14, 3.34) in favor of day 5 cryopreserved embryos (Table 3). When adjusting for age at cryopreservation and fresh cycle day 3 FSH, the implantation odds for day 5 transfers remained significantly higher compared to day 6 (OR: 1.91; 95% CI: 1.00, 3.67). The LBR also demonstrated a similar trend with an odds ratio of 1.18 for those blastocysts cryopreserved on day 5; however there was a wide 95% confidence interval (0.61, 2.30) when adjusting for age and day 3 FSH.

Discussion

The blastocyst stage remains the optimal time to cryopreserve embryos as the greater cell number and further differentiation results in a higher likelihood of surviving the freeze-thaw process (14). Blastocyst survival of more than 90% was demonstrated in our population and there were no differences between groups, suggesting that the time to attain the blastocyst stage does not impact the likelihood of cryopreservation survival. Therefore other factors must account for the differences in IR between groups.

The ability to develop supernumerary blastocysts predicts an improved cohort of developing embryos and predicts fresh cycle outcome (7). Yet the predictive value of cryopreserved embryos remains to be fully determined. The blastocyst developmental rate appears to affect the overall IR (15). Wilson *et al.* showed that day 5 expanded blastocysts had improved IR

(51% vs. 28%; $p<0.0001$) and clinical PR (70% vs. 42%; $p<0.0001$) when compared to day 5 non-expanded blastocysts (16). Fresh cycle day 5 blastocyst transfers resulted in a 2- to 5-fold increase in IR and a 2- to 3-fold increase in PR compared to day 6 transfers (9,17,18).

Despite fresh cycle data suggesting that the time to blastocyst development predicts cycle outcome, there remains inconsistent data regarding the importance of the rate of embryo development on FBET success. Behr and colleagues retrospectively examined 64 non-donor FBET cycles and were unable to find a difference among day 5 and day 6. However there was a relatively low IR (16%) (19). Our population demonstrated a significantly higher IR among the day 5 group (32.2%) as compared to day 6 transfer (19.2%). Despite IR differences between groups, a statistically significant difference when controlling for confounding variables was not observed for LBR. In order to achieve sufficient power (80%) to detect the 12.6% difference in LBR between groups as seen in our analysis, a prospective study would require approximately 180 patients in each arm (α -error=0.05). Due to the retrospective nature of the present study, the sample size was determined by the study interval. As a result there was insufficient statistical power to detect a difference in LBR. Nevertheless, a trend toward improved odds in the day 5 group was observed when adjusting for confounding variables signifying that the observations made for fresh cycles would apply to FBET cycles as well.

There remained important differences in pregnancy outcomes among groups. The day 5 group demonstrated a higher spontaneous abortion rate (9.6% vs. 2.7%; $p=0.01$). It may be speculated that this increased spontaneous abortion rate was a function of a higher overall IR. However, the spontaneous abortion rate in the day 6 group was much lower than the expected 8-9% seen for both day 5 and day 6 cryopreserved blastocyst transfers in other studies (14) which may in turn explain why the difference in clinical PR and LBR were not statistically different. Nevertheless, this study was not designed to assess fully this difference. However, we were able to demonstrate that the negative pregnancy rate was higher in the day 6 group compared to the day 5 groups (48.0% vs. 33.7%; $p=0.04$) reflecting an overall decrement in IR. This observation suggests that, like the outcomes of fresh ART cycles (15), the rate of embryo development does predict key outcomes and may indicate a more biologically competent cohort of embryos.

In summary, cryopreserved day 5 blastocysts have higher IR even when adjusting for potential confounding factors. While there was a significant improvement in IR for day 5 transfers, the LBR odds demonstrated a non-significant trend toward better outcome (OR:1.18) when controlling for age at cryopreservation and day 3 FSH. Successful pregnancies did occur utilizing cryopreserved embryos that did not achieve blastocyst stage until day 6, suggesting that the rate of embryo development has an important impact on cycle outcome but cannot fully account for all differences. Ultimately these results add further evidence to the notion that the rate of embryo development is associated with cycle outcome as demonstrated by improved implantation among cryopreserved embryos. While not discounting the value of cryopreserved day 6 blastocyst, one may extrapolate from this data that to optimize the IR for elective single embryo cryopreserved blastocyst transfers, practitioners may preferentially select those embryos demonstrating the more rapid development for transfer first.

Acknowledgements

Financial Support: This research was supported, in part, by the Reproductive Biology and Medicine Branch, NICHD, NIH, Bethesda, MD.

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Table 1
Patient characteristics during previous fresh ART cycle

	Cryopreservation on Day 5	Cryopreservation on Day 6	p-value
Patients (n)	100	72	
Age at cryopreservation (yrs ± SD)	32.6 ± 4.0	33.2 ± 3.6	0.28
Day 3 FSH (mIU/mL)	6.4 ± 2.1	6.8 ± 2.4	0.36
SART diagnosis			0.58
Endometriosis	3 (3%)	6 (8.3%)	
Tubal Factor	21 (21%)	28 (38.9%)	
Male Factor	38 (38%)	10 (13.9%)	
Anovulation	11 (11%)	8 (11.1%)	
Idiopathic	14 (14%)	12 (16.7%)	
Combined	13 (13%)	8 (11.1%)	
Insemination method (% ICSI)*	18 (18.4%)	12 (16.7%)	0.46
Number of embryos cryopreserved (n ± SD)	3.8 ± 2.3	3.5 ± 2.0	0.45
Use of sequential media (%)	96.0%	95.8%	0.96
Length of cryopreservation (yrs ± SD)	1.6 ± 1.5	1.4 ± 1.3	0.41
Day 3 ET (%)	33 (33.3%)	29 (40.8%)	0.30
Outcome of fresh cycle			
Clinical pregnancy (n, %)	52 (53.6%)	31 (43.7%)	0.21

* Excludes 2 patients undergoing IVF/ICSI split

Table 2
Frozen blastocyst embryo transfer cycle characteristics and outcome

	Transfer of day 5 group	Transfer of day 6 group	p-value
Age at transfer (yrs)	n=100	n=72	
Blastocysts Thawed (n)	34.0 ± 4.2	34.5 ± 3.8	0.45
Blastocysts Transferred (n)	2.4 ± 0.7	2.4 ± 0.8	0.78
Blastocyst survival (%)	2.2 ± 0.6	2.2 ± 0.6	0.79
Morphology of cryopreserved embryos transferred (n,%)	93	92	0.68
Expanded blastocysts	0 (0%)	1 (1.4%)	0.34
Blastocysts	99 (99.0%)	71 (98.6%)	
Early blastocysts	0	0	
Morula	1 (1%)	0 (0%)	
Implantation rate (%)	32.2%	19.2%	0.01
Pregnancy outcomes (%)			
Ongoing pregnancy/live birth	41 (43.3%)	23 (30.7%)	0.33
Biochemical pregnancy	13 (13.5%)	13 (18.7%)	0.29
Spontaneous abortion	13 (9.6%)	1 (2.7%)	0.03
No pregnancy	33 (33.7%)	35 (48%)	0.04

Table 3
Multivariate modeling for implantation and clinical pregnancy (using generalized estimating equations)

Outcome	Adjustments	OR [95%CI]
Implantation		
Model 1	Day 5 versus 6, unadjusted	1.96 [1.14, 3.34]
Model 2	Day 5 versus 6, adjusted for maternal age at cryopreservation	1.88 [1.10, 3.25]
Model 3	Day 5 versus 6, adjusted for maternal age at cryopreservation, day 3 FSH	1.91 [1.00, 3.67]
Clinical pregnancy		
Model 1	Day 5 versus 6, unadjusted	1.41 [0.74, 2.67]
Model 2	Day 5 versus 6, adjusted for maternal age at cryopreservation	1.35 [0.70, 2.57]
Model 3	Day 5 versus 6, adjusted for maternal age at cryopreservation, day 3 FSH	1.18 [0.61, 2.30]