Developmentally delayed cleavage-stage embryos maintain comparable implantation rates in frozen embryo transfers

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Abstract

Purpose In fresh IVF cycles, embryos reaching the eight-cell stage on day 3 of development are thought to have a higher chance of implantation than those reaching this stage on day 4. To determine whether this difference persists after cryopreservation, we compared pregnancy and implantation rates between frozen embryo transfer (FET) cycles using delayed cleavage-stage embryos (cryopreserved day 4) and normal cleavage-stage embryos (cryopreserved day 3).

Methods Participants underwent FET between 2008 and 2012 using embryos cryopreserved on either day 3 ($n=76$) or day 4 ($n=48$), depending on the length of time needed to achieve the eight-cell stage. All embryos, regardless of day of cryopreservation, were thawed and transferred on the 4th day of vaginal progesterone following endometrial preparation with oral estradiol. Chi-square and Mann-Whitney U tests were used to compare patient demographics and cycle outcomes.

Results More women in the day 4 group had diminished ovarian reserve (44 vs 16 %, $p=0.003$). Pregnancy outcomes in preceding fresh cycles were not different between the two

groups. Pregnancy, implantation, and live birth rates following FET did not differ between the day 3 and day 4 groups.

Conclusions This is the first study to address outcomes using day 3 versus day 4 cryopreserved embryos. Despite a higher prevalence of diminished ovarian reserve (DOR) in the day 4 group, delayed cleavage-stage embryos utilized in FET cycles performed as well as embryos growing at the normal rate, suggesting delayed embryo development does not affect embryo implantation as long as endometrial synchrony is maintained.

Keywords Frozen embryo transfer · Cleavage-stage embryo · Embryo cryopreservation · Delayed embryo development

Introduction

The first pregnancy conceived from the thaw and transfer of a cryopreserved embryo (frozen embryo transfer (FET)) was

Capsule Developmentally delayed cleavage-stage embryos that reach the eight-cell stage or better by day 4 have similar outcomes in frozen cycles to normal embryos when synchronized to endometrial development.

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reported in 1983 [1]. Since that time, the proportion of assisted reproductive technology procedures involving embryo cryopreservation has risen [2]. The pregnancy rates for these procedures have also improved, drastically increasing the number of FETs performed yearly. In 2012 alone, over 40,000 FET procedures were performed in the USA alone [3]. As cryopreserved embryos represent an ever-increasing proportion of embryos transferred, interest in identifying embryos with the greatest implantation potential in FET cycles also increases.

Multiple studies of embryo morphology have identified features indicative of better implantation potential in fresh cycles using cleavage-stage embryos [4–7]. Embryos with eight blastomeres or at compacting stage on day 3 generally have been shown to perform better than those with fewer blastomeres [5]. In fresh IVF cycles, the window of endometrial receptivity is set by the timing of oocyte retrieval and cannot be adjusted for embryos which are delayed in development. Cryopreservation of delayed but normal-appearing embryos affords the opportunity to resynchronize the developmental stage of the embryo with progression of endometrial luteinization and the window of receptivity.

Previous studies have assessed outcomes using frozen thawed blastocysts with delayed development. El Toukhy et al., in 2011 [8], compared embryos that reached the blastocyst stage on day 5 versus day 6, all of which were subsequently cryopreserved using slow freezing, thawed, and transferred on day 6 of progesterone. Comparable pregnancy and live birth rates were achieved in both groups. These results stand in contrast to an earlier, similar study by Levens et al. in 2008 showing a higher implantation rate in day 5 blastocysts than in day 6 blastocysts transferred on the 6th day of progesterone, although in this study, live birth rates were also similar between groups [9].

Both studies support the notion that embryos with delayed blastocyst formation can be cryopreserved and resynchronized with little impact on birth rates. However, to our knowledge, no studies to date have compared outcomes in cleavage-stage embryos with delayed development to those with normal development. Although many fertility providers have transitioned exclusively to the transfer of blastocyst-stage embryos, there remain embryology laboratories throughout the globe who continue to provide cleavage-stage embryo transfers in some or all of their patients. Therefore, the need to identify best practices when handling cleavage-stage embryos continues, so that these providers may continue to care optimally for their patients. The aim of this study was to compare outcomes in frozen embryo transfer cycles when using cleavage-stage embryos reaching the eight-cell stage on day 4 (delayed) to those who reached eight cells or better on day 3 (normal), when the endometrium is synchronized to the developmental stage at the time of embryo cryopreservation.

Materials and methods

The study was a retrospective observational cohort study of patients who underwent transfer of cryopreserved cleavage-stage embryos at USC Fertility between January 2008 and December 2012. Institutional review board approval was obtained from the University of Southern California Health Sciences Institutional Review Board prior to initiation of this study.

Patient selection

Eligible patients were identified through a search of the Society for Assisted Reproductive Technologies (SART) database. Only those patients who underwent transfer of cleavage-stage embryos cryopreserved on either day 3 or day 4 of development were included, and all frozen embryo transfers took place on the 4th day of progesterone administration. Both donor and autologous frozen embryo transfer cycles were included. Embryo transfers containing a combination of cleavage-stage embryos cryopreserved on both day 3 and day 4 were excluded. Embryos derived from intracytoplasmic sperm injection (ICSI) performed on day 2 oocytes derived from in vitro maturation were also excluded.

Embryo cryopreservation

When patients underwent fresh embryo transfer on day 3, all other eight-cell or better embryos were also cryopreserved on day 3. The remaining embryos were observed one additional day, and any additional eight-cell or compacting embryos were thus cryopreserved on day 4. Any remaining embryos were discarded. All embryos were slow frozen using 1.5 M propylene glycol (PROH) and 0.1 M sucrose (Irvine Scientific, Santa Ana, CA).

Frozen embryo transfers

Each patient underwent endometrial preparation beginning on the 2nd day of menstrual bleeding. Oral estradiol was initiated at 2 mg twice daily, increasing to three times daily after at least 1 week, and continuing for a minimum of five additional days at the increased dose. Prior to initiation of progesterone, each patient underwent ultrasound evaluation of endometrial thickness and ovarian follicle formation. If the endometrial thickness was deemed adequate (≥ 7 mm), serum progesterone was measured to ensure that ovulation had not occurred. Progesterone was then initiated by either the vaginal (200 mg three times daily) or intramuscular (IM, 50 mg twice daily) route, or in combination. Embryo transfer took place on the 4th day of progesterone administration.

Data collection

Once study candidates were identified, charts were reviewed for demographics, characteristics of the fresh cycle from which the embryos were obtained, and pregnancy outcomes. Demographics collected included female age at the time of embryo transfer, height, weight, ethnicity, gravity, parity, duration of infertility prior to presentation, and reason for infertility. Data were collected from SART data on the fresh cycle that produced the embryos utilized in the FET cycle of interest. Fresh cycle characteristics included the use of autologous versus donor oocytes, ovarian reserve testing (day 3 FSH and antral follicle count), total gonadotropin dose required, total oocytes retrieved, the number of 2pn embryos obtained, clinical pregnancy, and live birth. Information on ovarian reserve testing and total gonadotropin dose was only reported for participants using autologous oocytes. Study data were collected and managed using REDCap electronic data capture tools hosted at the University of Southern California [10].

Each participant's first frozen embryo transfer during the study period was included in the analysis. Frozen transfer cycle characteristics were recorded including the day of embryo cryopreservation, the number of embryos transferred, the number of gestational sacs at 6-week ultrasound, and the number of live-born infants beyond 24 weeks of gestation. Outcomes of interest were implantation, clinical pregnancy, and live birth rates, as well as live birth per embryo transferred. Implantation was defined as appearance of a gestational sac on ultrasound at 6 weeks of gestation and is described per embryo. Clinical pregnancy was defined as at least one fetal heart rate detected at 6 weeks of gestation, and live birth was the delivery of at least one viable infant at ≥ 24 weeks of gestation. Live birth per embryo was defined as the number of live-born infants divided by the number of embryos transferred.

Data analysis

Age and parity are reported as medians. Chi-square or Fisher exact tests were used to compare categorical variables, as appropriate. Continuous variables were compared using the non-parametric Mann-Whitney *U* test. Proportions were compared by calculating the *z*-ratio to determine the associated two-tailed probability. The significance level was set at $p \leq 0.05$ for all statistical tests. Sample size calculation revealed that 35 patients are needed in each group to have 80 % power to detect a 10 % difference in implantation rate between day 3 and day 4 embryos.

Results

A total of 344 patients underwent frozen embryo transfer during the study period. Two hundred twenty of these were

Table 1 Patient demographics

	Cryopreserved day 3 Normal N=76	Cryopreserved day 4 Delayed N=48	<i>p</i>
Age, years, median (range)	37 (23–52)	39 (29–54)	0.09
Parity, median (range)	0 (0–3)	0 (0–3)	0.21
Oocyte donor used (%)	19.7	25.0	0.49
Infertility diagnosis (%)			
Male factor	35.5	25.0	0.22
Tubal factor	11.8	10.4	0.81
Diminished ovarian reserve	32.9	58.3	0.005*
Diminished ovarian reserve Autologous oocytes only	16.4 (10/61)	44.4 (16/36)	0.003*
Anovulation	15.8	10.4	0.40
Endometriosis	6.6	12.5	0.26
Uterine factor	11.8	14.6	0.66
Situational	6.6	6.3	1.0
Unexplained	10.5	8.3	0.76

* $p < 0.05$, statistically significant

excluded; 179 were excluded either due to transfer of a combination of day 3 and day 4 embryos or because 2pn embryos were thawed and grown to cleavage or blastocyst stage. Thirty-eight transfers used embryos cryopreserved at blastocyst stage, and three additional transfers were excluded because day 4 embryos were thawed and transferred on day 5 of progesterone.

The remaining 124 patients met the study criteria, 76 with transfer of day 3 embryos (normal) and 48 with transfer of day 4 embryos (delayed). Donor oocytes were used in 27 participants, with 15 using day 3 embryos for FET and 12 using day 4 embryos. Table 1 compares patient demographics between groups overall. There were no differences between groups in median age or parity, and donor oocytes were utilized at a similar rate.

Significantly more women in the delayed group had a diagnosis of diminished ovarian reserve (DOR), defined as either early follicular FSH greater than 10 IU/L, antral follicle

Table 2 Autologous fresh cycles preceding the frozen embryo transfer included in this study

Fresh cycle characteristics, autologous (medians)	Cryopreserved day 3 Normal N=61	Cryopreserved day 4 Delayed N=36	<i>p</i>
Age, years, median (range)	35 (22–42)	35.5 (28–43)	0.20
BMI, kg/m ²	22	24	0.14
Day 3 FSH (IU/L)	6.5	6.5	0.97
Antral follicle count	17	13	0.07
Total gonadotropin dose (IU)	2475	4200	0.001*

* $p < 0.05$, statistically significant

Table 3 Outcomes of fresh cycles producing the FET cycles included in this study

Fresh cycle outcomes	Autologous			Donor		
	Cryopreserved day 3 Normal N=61	Cryopreserved day 4 Delayed N=36	<i>p</i>	Cryopreserved day 3 Normal N=15	Cryopreserved day 4 Delayed N=12	<i>p</i>
Number oocytes retrieved (median)	18	16.5	0.39	16	20.5	0.32
Number 2pn embryos (median)	10	9	0.12	7	11.5	0.07
Clinical pregnancy rate (%)	34.5 (20/58)	31.4 (11/35)	0.82	20.0 (3/15)	8.3 (1/12)	0.61
Live birth rate (%)	15.5 (9/58)	14.3 (5/35)	0.89	6.7 (1/15)	8.3 (1/12)	1.0

count ≤ 10 , or previous low oocyte yield of ≤ 3 . This was noted despite a similar prevalence of donor oocyte usage between groups (Table 1). Because all recipients of donor oocytes were presumed to have a diagnosis of DOR or ovarian failure, a second comparison of this infertility diagnosis was performed excluding donor cycles. Even when donor cycles were excluded, the delayed group still had a higher prevalence of DOR. Characteristics of the autologous fresh cycles that created the embryos used in the FET cycles compared in this study are reported in Table 2. The delayed group began with a lower antral follicle count that trended toward significance, and required significantly greater cumulative doses of gonadotropins during their fresh stimulation (Table 2).

Table 3 shows the outcomes of fresh embryo transfers performed during fresh stimulation cycles that produced the cohort of cryopreserved embryos used in this analysis. Not all of the 124 participants underwent prior fresh embryo transfer, as 4 participants cryopreserved all embryos (3 in the day 3 group, 1 in the day 4 group). In the day 3 “normal” group, 73 participants (58 autologous, 15 donor oocytes) had a prior fresh embryo transfer, as did 47 participants (35 autologous, 12 donor oocytes) in the day 4 “delayed” group. Clinical pregnancy rates were similar between the normal and delayed groups in preceding fresh cycles for autologous cycles (34.5 vs 31.4 %, $p=0.89$) and slightly higher for the normal group for donor cycles (20 vs 8.3 %, $p=0.61$), but this did not reach statistical significance.

Following FET, no difference was noted in implantation rate, clinical pregnancy rate, live birth rate, and live birth per

embryo between the normal and delayed groups, whether autologous or donor oocytes were utilized (Table 4). When adjusting for possible confounders among autologous cycles using a multiple regression model including age, AFC, and diagnosis of DOR, there remained no significant difference in clinical pregnancy rate ($p=0.73$) or live birth rate ($p=0.81$).

Discussion

In order for implantation to occur, an embryo with implantation potential must be present in the uterus during the window of endometrial receptivity. The success of frozen embryo transfer has been dependent on providers’ ability to synchronize endometrial progression to embryo development. In autologous fresh IVF cycles, the progesterone level rises toward the end of the follicular phase due to increased steroidogenesis by multi-follicular development in the ovary. This has been shown to cause accelerated endometrial maturation in the subsequent luteal phase [11]. Fresh transfer of delayed embryos into this accelerated endometrial environment would logically risk dyssynchrony between the embryo and the endometrium and possibly lead to a higher rate of implantation failures. As previously noted, embryos with fewer than eight blastomeres on day 3 have been shown to have lower implantation rates in fresh cycles [5]. In our study of frozen embryo transfers, delayed embryos did not display this lower implantation potential.

Table 4 Frozen embryo transfer outcomes

Frozen-thawed cycle outcomes % (n/total)	Autologous			Donor		
	Cryopreserved day 3 Normal N=61	Cryopreserved day 4 Delayed N=36	<i>p</i>	Cryopreserved day 3 Normal N=15	Cryopreserved day 4 Delayed N=12	<i>p</i>
Implantation rate	12.0 (20/166)	11.9 (12/101)	0.89	7.7 (3/39)	12.9 (4/31)	0.45
Clinical pregnancy rate	24.6 (15/61)	25.0 (9/36)	1.0	20.0 (3/15)	16.7 (2/12)	1.0
Live birth rate	21.3 (13/61)	19.4 (7/36)	1.0	20.0 (3/15)	8.3 (1/12)	0.61
Live birth per embryo	9.0 (15/166)	8.9 (9/101)	0.86	7.7 (3/39)	6.5 (2/31)	1.0

There may be different reasons for this. Delayed embryos were transferred on the 4th day of progesterone, the same day as normally developing embryos cryopreserved on day 3. In this manner, the delayed embryo is afforded one additional day of “catch-up” growth and then cryopreserved and subsequently transferred as though it had been cryopreserved on day 3. This effectively resynchronizes the embryo to endometrial development and may be responsible for the similar implantation, pregnancy, and birth rates observed among delayed embryos in our study. This principle likely also explains why embryos with day 6 blastocyst formation have been shown in previous studies to have similar implantation and pregnancy rates in frozen cycles to embryos forming blastocysts on day 5.

It is also possible that the retrospective nature of our study, as well as the sample size, would not be able to detect small differences in implantation rates between normal and delayed frozen cleavage-stage embryos. Since the outcomes were so similar between groups, with no trends toward statistical significance, we consider it more likely that no clinically significant differences exist. In a recent study by Zhao et al. [12], embryos with only four blastomeres on day 3 were shown to form blastocysts at lower rates, but those embryos that did reach the blastocyst stage and were cryopreserved performed equally well in thawed cycles to thawed blastocysts from embryos having more blastomeres on day 3. This study supports our findings that if given the opportunity to develop further, delayed cleavage-stage embryos showing further growth can perform well in FET cycles.

Arguably, the group undergoing transfer of delayed frozen embryos should represent a poorer prognosis group. The prevalence of DOR was higher in this group, and although statistical significance was not reached, this group also had a lower clinical pregnancy rate in preceding fresh embryo transfers. If the use of cryopreserved cleavage-stage embryos with delayed development did have a negative impact on pregnancy rates, this group should have been more sensitive to the effect. However, no difference was noted despite the increased incidence of DOR, making it unlikely that a true difference was missed in this study design. This study was not designed to answer whether women with DOR were more likely to have embryos with delayed development, but this is an interesting clinical question.

Although many providers have transitioned to cryopreservation of only embryos that reach blastocyst stage in culture, it is questionable whether this is the best practice for all patients. Women with advanced maternal age or those with prior failed IVF cycles may gain some benefit from the transfer of embryos from laboratory culture to the uterine environment at an earlier time point. These embryos may be particularly sensitive to differences in culture media and an incubator as compared to the uterine cavity, which, although not the normal physiologic location for embryos at day 3 of development, may represent a less detrimental environment.

Conclusions

This is the first study to address day 3 versus day 4 cryopreserved embryos. Cleavage-stage embryos that reach at least the eight-cell stage by day 4 of development appear to have the same implantation potential as embryos reaching the same stage by day 3. Cryopreservation of these delayed embryos and resynchronization to endometrial luteinization renders them similarly capable of implantation, clinical pregnancy, and live birth.

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