Time to blastulation is superior to individual components of embryo grading for live-birth prediction

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Objective: To compare components of the embryo grading system with time for blastocyst formation (i.e., day of embryo transfer) for predicting live-birth rate in frozen embryo transfer cycles.
Design: Retrospective cohort study.
Setting: University-affiliated fertility clinic.
Patient(s): From January 2015 to October 2018, 870 frozen embryos transferred in a total of 509 women and 728 cycles at our institution.
Intervention(s): None.
Main Outcome Measure(s): Probability of live birth per cycle.
Result(s): In unadjusted analysis of embryo grading components, both inner cell mass (ICM) and trophectoderm grades demonstrated a correlation with live-birth rates. However, this effect was lost in the ICM subgroup analysis by day of embryo transfer and preserved only in declining trophectoderm grades of day-6 transfers. In the adjusted analysis for prediction of live birth, only day of transfer was statistically significant. When assessing the composite score by Society for Assisted Reproductive Technology (SART) embryo grading, good embryos that blastulated on day 6 were statistically significantly less likely than day-5 embryos to result in live birth (risk ratio 0.70; 95% confidence interval, 0.58–0.85). Finally, in a predictive model adjusted for all individual components of embryo grade, the day of blastulation was the only statistically significant contributor.
Conclusion(s): Time to blastulation is superior to other individual components of embryonic grading for prediction of live birth. (Fertil Steril Rep® 2020;1:243–8. ©2020 by American Society for Reproductive Medicine.)
Key Words: Blastulation timing, embryo grading, FET

Despite ever-improving in vitro fertilization (IVF) techniques, live birth cannot be guaranteed even with presumed optimal embryo selection. Among other factors, morphologic grading guides embryo selection for transfer. The major components of embryo formation are considered for grading: degree of blastocyst expansion, inner cell mass (ICM), trophectoderm (TE), and day of blastulation (day 5 or 6). The Society for Assisted Reproductive Technology (SART) subclassifies embryos based on the grades of ICM and TE into good, fair, and poor (1). Several studies have shown that embryo grade is predictive of live birth (1–6). However, studies have differed with regard to whether the weight of different components is equal or one component may be more predictive than others.

Although some studies in fresh embryo transfers have designated TE as the single best predictor of live birth (4, 5), these did not consider timing of blastulation in their multivariate analysis. Further, in an era of increasing use of frozen embryo transfer (FET), this has not been demonstrated consistently in FET cycles. In one of the largest studies examining this question, Bakkensen et al. (7) described a trend in pregnancy and live-birth rates (LBR) by successive TE and expansion scores. However, despite this trend, after adjusting for age and body mass index (BMI), no statistically significant impact on the relative risk of live birth was noted for TE. Also remarkable was that the day of blastulation was not found to be a statistically significant contributor to their predictive model. In contrast, Irani et al. (8) demonstrated in their analysis of 417

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euploid FET cycles that ICM was the single best predictor of ongoing pregnancy rate, but LBR was not reported. In this analysis, the impact of TE on outcomes diminished in the adjusted analysis, as it had in the Bakkensen study. While these data were adjusted for day of blastulation, they did not report on the individual effect on the model. Desai et al. (6) reached yet another conclusion in the analysis of FET cycles when they determined that delayed blastulation and blastocyst expansion were the greatest predictors of live birth. In their assessment, embryo grade after warming was used, and the multivariate model did not include ICM or TE grade.

Studies examining this question have varied in their statistical approach, outcomes of interest, and inclusion of other embryo morphology features, leading to mixed conclusions in the literature. We assessed the predictive value of different components of embryo morphology, along with blastulation timing, in a fully adjusted model examining LBR after FET cycles.

MATERIALS AND METHODS

All patients who underwent FET between January 2015 and October 2018 at UNC Fertility were candidates for inclusion in this study. These patients were identified by query of the electronic medical records after obtaining approval from the institutional review board. Patients were excluded if they underwent cleavage-stage embryo transfer, if their embryos were from an outside institution, if they were lost to follow-up evaluation with an unknown cycle outcome, or if they had multiple embryo transfers and an unequal number of live births (requiring an "all or nothing" outcome to be retained).

All patients underwent controlled ovarian hyperstimulation with gonadotropins, using either gonadotropin-releasing hormone (GnRH) agonist down-regulation, GnRH flare, or GnRH antagonist protocols. Oocyte maturation was triggered with GnRH agonist or human chorionic gonadotropin (hCG) when two or more follicles reached a mean diameter $\geq$18 mm, and oocyte retrieval performed 35 hours later. Oocytes were inseminated by intracytoplasmic sperm injection (ICSI) or conventional insemination.

Embryos were subsequently cultured in continuous single culture media (Irvine Scientific Fujifilm) supplemented with 10% serum protein substitute (Cooper Surgical). Embryos were cultured in Planer (Cooper Surgical) or Miri (Esco) incubators through day 5 or 6 of development. Embryo grading was performed by embryologists in accordance to the Gardner and Schoolcraft embryo grading system (9) on final assessment of the blastocyst before cryopreservation. Three embryologists were involved in the grading of blastocysts in this data set, and annual quality control reviews are performed to ensure consistency among the embryologists in our facility. Blastocysts with a grade of 3BB or better were cryopreserved on day 5 of development. Blastocysts with a lower grading were cultured for an additional day. Blastocysts with a grade of 3CB or 3BC or better were cryopreserved on day 6 of development. Cryopreservation was performed via vitrification using high security vitrification straws (Irvine Scientific FujiFilm) and Irvine Scientific vitrification medium and protocol. Embryo thawing was also performed using Irvine Scientific vitrification thawing medium.

All cycles were programed FET cycles with endometrial preparation with vaginal or oral estradiol, followed by vaginal or intramuscular progesterone for 5 days before embryo transfer. The endometrial lining was required to be $\geq$7 mm to proceed with a transfer. Embryos were selected for transfer by prioritizing the day of blastulation, followed by expansion, trophectoderm, and ICM, respectively. Other specifics of oocyte stimulation and FET preparation protocols were at the discretion of individual providers.

For the composite analysis, participants' precryopreservation embryos were classified based on SART grading as either good, fair, or poor (Supplemental Table 1, available online). Multiple embryo transfers were included in analysis only if the cycle outcome produced an outcome for each embryo (either not pregnant, or number of live-born children equaled the number of embryos transferred) to account for a definitive outcome per individual embryo. As such, 124 included cycles were double-embryo transfers and five cycles with triple-embryo transfers, but each embryo outcome was assessed individually. Subgroup analysis was also performed on single-embryo transfer cycles. Demographic and cycle characteristics were collected, including age, BMI, infertility diagnosis, blastulation timing, and use of preimplantation genetic testing for aneuploidies (PGT-A). Fifty-five FET cycles were from donor egg–derived embryos, and the age of the donor at time of retrieval was used in the analysis to account for this.

All analyses were conducted in SAS 9.4 (SAS Institute). Missing data were excluded from the analysis. Univariate and bivariate demographics were assessed among women at their first embryo transfer. *P* values were calculated using chi-square tests. The proportion of cycles that resulted in a live birth were tabulated based on each grading level for expansion, ICM, and trophectoderm, further stratified by day of embryo transfer. We conducted tests of linear trend evaluating the association between levels of ICM and trophectoderm in association with proportion of cycles resulting in a live birth. Expansion was treated nominally, and a chi-square test was performed to identify any statistical differences in live-birth proportion by expansion level. All tests were conducted within day of blastulation. These analyses were unadjusted and measured crude proportions of cycles resulting in live birth.

For adjusted models, potential confounders were identified using a directed acyclic graph and included maternal age in years (continuous), BMI (kg/m^2, continuous), PGT, and SART infertility diagnosis (nominal). All variable data were collected from the electronic medical records. The SART diagnosis was subsequently excluded from future models as it did not meaningfully affect the estimates. A composite variable was created using SART grading and day of transfer. Due to small sample size, gradings of "poor" were excluded from composite analysis. As odds ratios are known to overestimate risk ratios in the presence of nonrare outcomes, risk ratios (RR) were selected for our adjusted analysis. We used log-binomial estimation to evaluate the "risk" of live birth across grading scores. Due to convergence issues, PROC

TABLE 1

Demographic factors at first cycle stratified by SART embryo grading.

		SART embryo grade			
Demographics	**Overall (n = 509)**	**Good (n = 477)**	**Fair (n = 29)**	**Poor (n = 3)**	***P* value**
Age (y)	32.8 (± 4.6)	32.7 (± 4.5)	33.4 (± 5.7)	38.3 (± 3.2)	.08
BMI (kg/m^2)	25.7 (± 5.8)	25.7 (± 5.8)	25.2 (± 5.3)	33.5 (± 10.1)	.06
Missing	3	2	1	0	
Infertility diagnosis					.64
Unexplained	111	104 (93.7)	6 (5.4)	1 (0.9)	
Endometriosis	26	25 (96.2)	1 (3.9)	0 (0)	
Ovulatory dysfunction	115	102 (88.7)	12 (10.4)	1 (0.9)	
Tubal factor	57	53 (93.0)	4 (7.0)	0 (0)	
Male factor	150	143 (95.3)	6 (4.0)	1 (0.7)	
Uterine factor	5	5 (100)	0 (0)	0 (0)	
Other	39	39 (100)	0 (0)	0 (0)	
Missing	6	6	0	0	
Day of blastulation					< .01
5	382	369 (96.6)	11 (2.9)	2 (0.5)	
6	127	108 (85.0)	18 (14.2)	1 (0.8)	
PGT[a]					.04
Yes	83	78 (94.0)	3 (3.6)	2 (2.4)	
No	426	399 (93.7)	26 (6.1)	1 (0.2)	
Progesterone route					.68
Vaginal	117	111 (94.9)	6 (5.1)	0 (0)	
Intramuscular	334	313 (93.7)	19 (5.7)	2 (0.6)	
Missing	58	53	4	1	

Note: Values are number and percentage or mean ± standard deviation where appropriate. BMI = body mass index; PGT = preimplantation genetic testing; SART = Society for Assisted Reproductive Technology.

[a] These numbers reflect first cycle analysis only. Due to subsequent use of PGT, 104 unique women underwent PGT in 150 cycles resulting in 161 embryos transferred.

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GENMOD's Poisson regression with robust variance was used. The modified Poisson estimates have been shown as valid on average, though lacking in efficiency (10). In these models, generalized estimating equations were used to account for nonindependence between women with multiple cycles.

Finally, we ran models with all exposures and covariates of interest to examine the strongest contributors to live birth, accounting for other included factors. Modified Poisson estimates using generalized estimating equations were used to account for patients with multiple cycles included in our analysis.

RESULTS

Five hundred and forty-seven women underwent FET during the identified time period. We ultimately included 509 women and 870 transferred embryos in the analysis in 728 cycles, after applying exclusion criteria. The demographic data are summarized in Table 1, subdivided by the composite embryo SART grade. The mean overall age was 32.8 (± 4.6) years. The BMI and infertility diagnosis were not different between the SART grades. Delayed blastulation (day 6) correlated with embryo grade and had a statistically significantly higher proportion of lower-grade embryos than the day-5 embryos, respectively. A total of 104 unique patients (20.4%) underwent PGT-A, with transfer of euploid embryos performed in these cases. Among all embryos transferred, 779 (89.5%) were classified as good quality (by composite SART grade), reflecting the prioritized method of embryo selection for primary transfer; 88 (10.1%) were designated as fair quality, and 3 (0.3%) were poor quality.

Univariate analysis (Supplemental Table 2, available online) showed that ICM and trophectoderm exhibited a linear decline in live birth with decline in score. Expansion did not appear to demonstrate a linear correlation, with the highest LBR observed for expansion of 4, 47% (P=.67). Live birth rate for an ICM grade of A was 51%, for B was 36%, and for C was 21% (P< .01). However, when these cycles were stratified based on blastulation timing (Table 2), only trophectoderm grade of day 6 embryos correlated with live birth.

Subsequent analysis was performed to assess the effect of composite morphologic grading and timing of blastulation, represented by SART composite scores of good, fair, and poor, using day-5 good-quality embryos as the referent (Table 3). When adjusting for age and BMI a priori, good-quality embryos that blastulated on day 6 exhibited a statistically significant relative reduction in LBR by 30% (RR 0.70; 95% confidence interval [CI], 0.58–0.85) compared with good-quality embryos on day 5. A statistically significant reduction is also seen in day-5 fair-quality embryos versus day-5 good-quality embryos (RR 0.61; 95% CI, 0.42–0.90).

Finally, log-binomial modeling was performed to evaluate the contribution of each characteristic of embryo morphology to likelihood of live birth. When accounting for all other covariates, as well as age and BMI, only day of blastulation and known euploid status were statistically significant predictors of live birth. The overall relative risk of live birth after transferring a day-6 embryo versus day-5 embryo was 0.73, (95% CI, 0.60–0.89), with statistical significance

TABLE 2

Proportion of in vitro fertilization cycles resulting in live birth by morphology component, subdivided by day of embryo transfer.

	Day 5			Day 6		
Component	**Grade**	**Live birth**	***P* value**	**Grade**	**Live birth**	***P* value**
Expansion (n = 870)						
	3 (n = 140)	0.32	.21	3 (n = 47)	0.28	.54
	4 (n = 382)	0.52		4 (n = 121)	0.30	
	5 (n = 89)	0.56		5 (n = 69)	0.30	
	6 (n = 8)	0.50		6 (n = 14)	0.29	
Inner cell mass (n = 870)						
	A (n = 354)	0.54	.82	A (n = 75)	0.36	.17
	B (n = 257)	0.40		B (n = 155)	0.29	
	C (n = 8)	0.50		C (n = 21)	0.10	
Trophectoderm (n = 868)						
	A (n = 286)	0.58	.07	A (n = 76)	0.33	.01
	B (n = 308)	0.42		B (n = 134)	0.29	
	C (n = 24)	0.17		C (n = 40)	0.25	

Note: P values for inner cell mass and trophectoderm reflect test of linearity. *P* value for expansion reflects a Mantel-Haenszel chi-square.

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TABLE 3

Risk ratios (95% confidence interval) for the association between embryo grading and day and live birth.

Day and grade	**Unadjusted (n = 867)**	**Adjusted (n = 860)[a]**
D5 Good (589)	Reference	Reference
D5 Fair (28)	0.62 (0.41–0.94)	0.61 (0.42–0.90)
D6 Good (190)	0.72 (0.60–0.87)	0.70 (0.58–0.85)
D6 Fair (60)	0.74 (0.54–1.01)	0.75 (0.56–1.00)

Note: The n values correspond to number of embryos included in the analysis.
[a] Adjusted risk ratios of live birth by composite SART grade and day of embryo transfer. Analysis adjusted for body mass index, age (continuous), and preimplantation genetic texting.

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maintained whether PGT-A was performed or not (Table 4). When the subgroup analysis was performed on single-embryo transfers (Supplemental Table 3, available online), this finding remained consistent for embryos without PGT-A testing. These findings suggest that prediction of performance of an embryo are largely driven by day of blastulation and known euploidy status.

DISCUSSION

Our findings indicate that day of blastulation may be preferential to guiding embryo selection over other individual morphologic grading components for FET cycles. Although known euploid status is a principal determinant of embryo selection, we found that day of blastulation is also a significant factor in predicting LBR. As age and BMI track with IVF outcomes (11, 12), in models that control for these variables, only day of blastulation remains a statistically significant factor in predicting live birth after FET. Composite scoring by the SART embryo grading system suggests greater predictive value for live birth than was seen by assessing or weighting individual components.

We attempted to discern the role of morphology for embryos that did not undergo PGT-A. Some time-lapse studies have argued that time to developmental landmarks are themselves indicative of implantation potential, but debate persists regarding the biological drivers of this (13). Multiple studies have suggested a correlation between delayed blastulation and aneuploidy (14–16). One study by Minasi et al. (15) demonstrated differences in expansion, TE and ICM grade, and blastulation timing based on aneuploidy. In addition, Lee et al. (16). compared morphokinetic features of embryos in conjunction with chromosomal assessment and demonstrated altered morphokinetics in aneuploid and high-level mosaic embryos. Specifically, these embryos exhibited longer times to achieve developmental landmarks, and most notably experienced delayed blastulation. Our finding of delayed blastulation having the greatest correlation and predictive value of live birth in a cohort of largely genetically untested embryos is supported by this earlier literature. We hypothesize that in adjusted models aneuploidy more greatly affects the timing of blastulation than other components and may explain why we do not see the impact of other components noted by Minasi et al. (15) in unadjusted analysis. Given that euploidy is considered the single greatest predictor of live birth (17), it follows that a possible marker associated with aneuploidy (e.g., blastulation rate) is of greatest value in embryos that did not otherwise undergo PGT-A.

It has also been proposed that delayed blastulation results in poorer outcomes secondary to embryo and endometrium dyssynchrony. In a study by Franasiak et al. (18) investigating attempted correction of dyssynchrony, poorer performance of day-5 and day-6 morphologically identical embryos were noted in fresh cycles. This reduced potential was only partially improved by conversion to FET. We have again demonstrated the impaired potential of these embryos in cryopreserved cycles, indicating an inherit reduction in embryonic quality of these embryos, unrelated to induced dyssynchrony by ovarian stimulation.

The strengths of our study include the large cohort of frozen embryo transfers. Additionally, the large proportion of patients who did not elect for PGT-A in this study broadens

TABLE 4

Risk ratios (95% CIs) for model evaluating live birth, stratified by PGT.

	All embryos		Adjusted among embryos	
Period	Unadjusted (n = 870)	Adjusted (n = 861)[a]	With PGT (n = 159)	Without PGT (n = 702)
Expansion				
3	0.90 (0.79–1.02)	0.91 (0.79–1.04)	1.13 (0.61–2.11)	0.93 (0.81–1.06)
4	Ref	Ref	Ref	Ref
5	1.15 (0.96–1.37)	0.95 (0.74–1.22)	0.99 (0.61–1.60)	1.04 (0.72–1.50)
6	1.09 (0.76–1.56)	1.00 (0.69–1.46)	1.33 (0.77–2.32)	0.38 (0.10–1.46)
Day of blastulation	0.87 (0.72–1.05)	0.73 (0.60–0.89)	0.64 (0.43–0.97)	0.76 (0.61–0.96)
ICM	0.90 (0.80–1.01)	0.97 (0.86–1.09)	0.99 (0.67–1.45)	0.94 (0.83–1.07)
Trophectoderm	0.90 (0.81–1.00)	0.95 (0.84–1.06)	0.98 (0.69–1.39)	0.92 (0.80–1.04)
PGT				
All	1.78 (1.51–2.11)	1.52 (1.19–1.95)		
None	Ref	Ref		

Note: ICM and trophectoderm were treated ordinally. ICM = inner cell mass; N = number of embryos included in the analysis; PGT = preimplantation genetic testing; Ref = reference value.
[a] Adjusted for coexposures, body mass index, and age.

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the applicability of these results in embryo selection for populations for whom preimplantation testing is not routinely pursued, such as a younger population. Further, we were able to expand on the work of Irani et al. (8) by reporting LBR as the outcome of highest clinical value (rather than intermediate outcomes such as clinical pregnancy rates). We also present composite analysis by the simplified SART classification system.

There are a few limitations to consider within our study. To assess outcome per embryo, multiple embryo transfers that did not result in an "all or nothing" outcome, where we could correctly correlate an outcome to its embryo of origin, were excluded. This disallowed assessment of the contribution of multiple embryo transfer to the likelihood of a single live birth, but we believe the restriction to all-or-nothing response may alleviate the need for this assessment overall. The maintained significance of day of blastulation on live birth in the subgroup analysis of genetically untested, single-embryo transfers supports that this impact is not confounded by multiple-embryo transfers. Although day of blastulation became less statistically significant in PGT-A–tested single-embryo transfers, this may be the result of underpowering due to a small sample size. However, it may also reflect the possible overlap of day of blastulation and euploidy, as discussed previously.

An additional limitation is that fair-quality embryos were often transferred secondarily, resulting in fewer fair embryos in comparison with good embryos. Similarly, the very low number of poor-quality embryo transfers performed by our clinic precluded accurate assessment of this composite group within this analysis. Finally, we are unable to adjust for parity or recurrent implantation failure (RIF) or pregnancy loss (RPL) in this database. However, previously published studies have not proved that parity contributes significantly, and RIF and RPL constitute a relatively small proportion of IVF recipients. Future larger studies may allow researchers to distinguish relatively small effects of different embryo morphological features on LBR.

CONCLUSION

After euploidy, delayed blastulation is the strongest embryonic predictor of embryo transfer success in FET cycles. Individual components did not appear to statistically significantly correlate with outcomes in the adjusted analysis. Composite scoring may offer improved predictive value but is limited in the study of FET given existing sequential embryo selection biases limiting the overall numbers of lower grade embryos transferred. Consideration should be given to weighing blastulation timing more highly than other individual morphologic grading components in cryopreserved embryo selection for transfer.

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