



Association between blastocyst morphology and pregnancy and perinatal outcomes following fresh and cryopreserved embryo transfer

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Abstract

Purpose To assess the importance of each blastocyst morphological criteria with pregnancy and perinatal outcomes.

Methods This single-center retrospective cohort study included blastocyst single embryo transfers (SET) performed between 1/2012–2/2018. Poisson regression was used to evaluate pregnancy outcomes following fresh and cryopreserved embryo transfer (CET) for association with blastocyst expansion, inner cell mass (ICM) quality, and trophoctoderm (TE) quality. Among cycles resulting in live birth, associations with preterm birth, small for gestational age (SGA) and large for gestational age (LGA), were evaluated using logistic regression.

Results A total of 1023 fresh and 1222 CET cycles were included, of which 465 (45.1%) fresh and 600 (48.5%) CET cycles resulted in singleton live birth. Clinical pregnancy rates increased with increasing expansion among fresh transfers (p for trend = 0.001) but not CET ($p = 0.221$), and with TE quality for both fresh and CET cycles ($p = 0.005$ and < 0.0001 , respectively). Live birth rates increased with increasing expansion (fresh $p = 0.005$, CET $p = 0.018$) and TE quality (fresh $p = 0.028$, CET $p = 0.023$). ICM grade was not associated with pregnancy outcomes; however, higher ICM quality among CET cycles was associated with increased chance of preterm birth ($p = 0.005$).

Conclusions In blastocyst SET, blastocyst expansion and TE quality were each associated with clinical pregnancy and live birth. While higher ICM quality was associated with increased chance of preterm birth among CET, no other associations with perinatal outcomes were identified. Clinicians can be reassured that pregnancies from blastocysts with lower expansion, ICM, or TE qualities are not more likely to result in adverse perinatal outcomes.

Keywords Blastocyst morphology · Perinatal outcomes · Birthweight · Preterm birth · In vitro fertilization

Introduction

Historically, the transfer of multiple embryos during in vitro fertilization (IVF) has been used to increase the chance of a successful implantation and clinical pregnancy. With the known risks that such practices impart for multifetal

pregnancies, guidelines have advocated for limiting the number of embryos transferred [1]. These restrictions have markedly increased the practice of single embryo transfer (SET) and have heightened the importance of the embryo selection process in identifying those embryos with the highest potential for implantation and healthy live birth.

The most widely used method for blastocyst evaluation is the morphologic grading system first described by Gardner and Schoolcraft [2] which evaluates blastocysts on three morphologic characteristics: degree of blastocyst expansion and hatching, size and compactness of the inner cell mass (ICM), and cohesiveness and number of cells of the trophoctoderm (TE). Previous studies have verified this system, confirming that the combination of these three characteristics is associated with the likelihood of implantation [3–6] and live birth [7–12]. However, which of the three characteristics is most associated with viability has been less well established. While it was

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traditionally thought that the ICM was most predictive of pregnancy and live birth, a number of recent studies have suggested that TE quality may in fact be most associated with viability [6–10]. A more thorough understanding of these individual associations is critically important in determining how to weight each characteristic for embryo selection, particularly in the era of single embryo transfer (SET).

The association between embryo morphology and perinatal outcomes has been less well studied. Small retrospective studies have found no correlation between embryo quality and perinatal outcomes [13, 14]; however, these studies did not analyze degree of expansion, ICM quality, and TE quality individually and therefore did not address potential independent associations of any one of these characteristics with perinatal outcomes. The few studies to date which have attempted to analyze the downstream fetal effects of each morphologic characteristic individually have been small and have yielded conflicting results, with one study suggesting a link between lower ICM grade and lower birthweight [15], and others finding no such association [16, 17]. These discrepant results highlight the need to further clarify these associations.

The goal of this study was to assess the association between each morphologic characteristic and clinical pregnancy and live birth, and for those cycles resulting in live birth, to assess the associations between each characteristic and perinatal outcomes including incidence of preterm birth (PTB), small for gestational age (SGA) and large for gestational age (LGA). We hypothesized that more advanced expansion and higher grades of TE and ICM would each be associated with an increased likelihood of clinical pregnancy and live birth and a decreased chance of adverse perinatal outcomes.

Materials and methods

This study was approved by the Partners Human Research Committee at the Brigham and Women's Hospital (Protocol #2018P000317). All data was retrieved from standard records collected during clinical care, thus informed consent was not required.

This was a retrospective cohort study of women undergoing fresh or cryopreserved day 5 or day 6 single embryo transfer (SET) at the Brigham and Women's Hospital from January 1, 2012, to February 28, 2018. During this time period, the culture system (culture media, gas tension, and incubators) was unchanged, as also were our laboratory standard operating procedures and the embryology team with the exception of one individual of nine. All embryologists undergo external proficiency testing bi-annually and internal competency evaluations annually.

Data were collected from our prospectively maintained departmental database and the hospital electronic medical record system. In vitro fertilization cycle and embryology data are

entered into the departmental database prospectively by clinicians and embryologists, respectively, and are audited for accuracy weekly.

Cycles which utilized preimplantation genetic testing or slow frozen embryos or resulted in monozygotic twins as well as cycles among women with recurrent pregnancy loss or uterine factor infertility were excluded from the analysis.

Ovarian stimulation

Stimulation protocols for fresh embryo transfer cycles utilized gonadotropin-releasing hormone (GnRH) antagonists, GnRH agonists for downregulation, estradiol priming or low-dose GnRH agonist flare protocols [18–21]. For CET cycles, both natural and programmed protocols were utilized, with the aim to achieve an endometrial thickness of 7 mm by ultrasound. The day of embryo transfer was determined according to internal protocols and patient history. In the majority of CET cycles, luteal support was provided with intramuscular progesterone in oil (locally compounded at Village Fertility, Waltham, Massachusetts, or Freedom Fertility, Byfield, Massachusetts), initiated at a dose of 25–100 mg per night, starting in the evening 5 days prior to transfer. In cases in which the serum level was below 20 ng/mL on the day of transfer, the dose of progesterone was increased by 25 mg. In those patients with adverse reactions to intramuscular progesterone, one applicator (90 mg) of vaginal progesterone gel (Crinone® 8%, Actavis Pharma, Inc.) was prescribed twice daily. The majority of patients undergoing fresh embryo transfer used vaginal progesterone gel (one applicator daily), beginning 2 days after oocyte retrieval for luteal support. Regardless of fresh or cryopreserved transfer, luteal phase support was continued through 10 weeks of gestation.

Laboratory protocols

Following retrieval, oocytes were either inseminated in groups (3–5 oocytes) or underwent intracytoplasmic sperm injection (ICSI) using routine procedures. A fertilization check was then performed at 16–18 h and zygotes with 2 pronuclei were cultured individually in 25- μ L drops of global total medium (Life Global Group, Cooper Surgical; Guildford, CT) overlain with mineral oil in *Esco Miri*® Benchtop multi-room incubators in a dry atmosphere consisting of 5% O₂ and 6–7% CO₂, balanced with N₂. The pH was controlled by taking weekly measurements per our standard operating procedure. The target pH in our laboratory is 7.30 \pm 0.5. The CO₂ of each incubator is adjusted to achieve this target. On day 3, embryos were moved to fresh drops of global total medium and on day 5 were either transferred fresh, cryopreserved, or cultured for a further 24 h for re-evaluation of cryopreservation suitability on day 6. Blastocysts that were transferred fresh or cryopreserved on day 5 or 6 were included in the study cohort.

Blastocyst cryopreservation

Blastocysts were cryopreserved individually using a Cryolock vitrification device (Irvine Scientific, Santa Ana, CA) as recommended according to the manufacturer specifications and warmed using the recommended warming protocol.

Blastocyst scores and grading

All embryos were evaluated and scored on day 5 and, if culture continued to day 6, again on day 6. Assessment of fresh embryos typically occurred within 2 h of transfer. Assessment of warmed embryos occurred immediately post-warming and again just before transfer. The expansion grade recorded for warmed embryos was that immediately prior to transfer. Per our laboratory protocols and to minimize the duration that embryos are out of the incubator, all embryos were evaluated by only one embryologist. A modification of the Gardner grading system was used as follows: each blastocyst was assessed for stage based on degree of expansion and quality of the ICM and TE. Blastocyst stage was graded as 5 through 8, where stage 5 is an embryo in which the blastocoele occupies more than half the volume, with some expansion in overall size and the zona pellucida (ZP) beginning to thin; stage 6 is an embryo in which the blastocoele completely fills the embryo and the ZP is not completely thinned; stage 7 is an embryo in which the blastocoele is completely filled, the embryo is fully expanded and the ZP is very thin; stage 8 is a hatching blastocyst in which the TE is starting to herniate through the ZP. The ICM and TE were each assessed for quality using grades of A–D. An ICM with “A” grade is prominent with many cells that are compacted and tightly adherent; an ICM with a “B” grade is discernible but has fewer cells that are loosely adherent; an ICM with a “C” grade has few discernible cells that are either compacted or loose and that may be difficult to distinguish completely from the TE; and an ICM assessed as grade “D” is characterized by either no discernible cells in any focal plane or cells that appear degenerate or necrotic. A TE with a grade “a” is characterized by a continuous layer of small uniform eye-shaped cells bordering the blastocoele; a grade “b” TE has fewer, larger cells that may not form a continuous layer; a grade “c” TE exhibits sparse cells that may be large; a grade “d” TE exhibits exclusively degenerating and/or absent cells.

Pregnancy and perinatal outcomes

Our primary outcomes were clinical pregnancy, defined as a gestational sac documented by ultrasonography at 7–8 weeks’ gestation, and live birth. Secondary outcomes included PTB, defined as delivery before 37 weeks’ gestation, and SGA and LGA (birthweight \leq 10th percentile and \geq 90th percentile, respectively). Notably, birthweight analyses were adjusted for

sex and gestational age through the conversion of raw birthweights to birthweight categorizations (*z*-scores) for each neonate using published data derived from US birth certificates corrected for sex and gestational age [22]. Live birth and perinatal outcomes data were derived from the electronic medical record or, for patients having delivered at outside institutions, were self-reported in follow-up communication with patients after birth.

Statistical analysis

For each morphologic characteristic, Poisson, logistic, or linear regression analyses were used as appropriate to estimate the relative risk (RR) or beta coefficient (β) and 95% CI as appropriate for pregnancy and perinatal outcomes by morphologic grade. A *p* value of < 0.05 was considered a statistically significant result. Analyses for each characteristic were adjusted a priori for the other two morphologic characteristics such that the effect of each was assessed independently of the other two. All analyses also controlled a priori for patient age at oocyte retrieval and maternal body mass index (BMI). Mean value substitution was used for missing values for patient BMI ($n = 8$). Birthweight analyses controlled a priori for infant sex and gestational age at delivery, as above. Additional covariates that were tested as potential confounders of the relationship between morphologic grade and pregnancy and perinatal outcomes included gravidity, parity, stimulation protocol, trigger type and peak estradiol (fresh cycles only), endometrial thickness, use of ICSI, day of embryo transfer (day 5 vs. day 6), and type of luteal support (vaginal vs. intramuscular progesterone); none of these variables confounded the effect estimates by more than 10% and so were not included in the final adjusted model [23]. Additionally, pre-existing maternal hypertension and diabetes were tested as potential confounders of the relationship between morphologic grade and birthweight but did not meet criteria for inclusion in the adjusted model. Fresh and CET cycles were analyzed separately. Statistical analyses were performed using SAS software version 9.4 [24].

Results

A total of 1023 fresh and 1222 CET cycles were included, of which 465 (45.1%) fresh and 600 (48.5%) CET cycles resulted in singleton live births.

Demographic and cycle characteristics of the study population as well as the distributions of our blastocyst cohorts among the three morphologic characteristics (expansion, ICM quality, and TE quality) are presented in Table 1, stratified by cycle type and live birth outcome. Notably, blastocyst morphology grades were consistently lower among CET cycles as compared with fresh cycles for all three characteristics,

Table 1 Patient demographics and cycle parameters for fresh and cryopreserved embryo transfers by live birth outcome

	Fresh <i>n</i> = 1023		CET <i>n</i> = 1222	
	Live birth <i>n</i> = 465	No live birth <i>n</i> = 558	Live Birth <i>n</i> = 600	No live birth <i>n</i> = 622
Patient age at retrieval (years)	32.0 ± 3.5	32.3 ± 3.5	32.4 ± 3.3	32.9 ± 3.5
Patient age at transfer (years) ^a	NA	NA	34.7 ± 3.9	34.9 ± 3.7
Carrier race/ethnicity				
Caucasian	333 (71.6%)	383 (68.6%)	420 (70.0%)	413 (66.4%)
African American	22 (4.7%)	30 (5.4%)	28 (4.7%)	43 (6.9%)
Hispanic	14 (3.0%)	27 (4.8%)	24 (4.0%)	18 (2.9%)
Asian	65 (14.0%)	69 (12.4%)	83 (13.8%)	97 (15.6%)
Native American	3 (0.6%)	1 (0.2%)	1 (0.2%)	1 (0.2%)
Other/declined	28 (6.0%)	48 (8.6%)	44 (7.3%)	50 (8.0%)
Carrier BMI (kg/m ²)	25.0 (5.5)	25.7 ± 6.0	25.2 (6.1)	26.1 ± 6.8
< 18.5	14 (3.0%)	9 (1.6%)	15 (2.5%)	15 (2.4%)
18.5–24.9	275 (59.4%)	326 (58.6%)	376 (62.8%)	354 (57.4%)
25–29.9	103 (22.2%)	114 (20.5%)	111 (18.5%)	112 (18.2%)
30+	71 (15.3%)	107 (19.2%)	97 (16.2%)	136 (22.0%)
Gravidity (<i>n</i>)	1.3 ± 1.0	0.6 ± 1.1	1.4 ± 1.2	1.1 ± 1.4
Parity (<i>n</i>)	0.2 (0.5)	0.2 ± 0.5	0.5 (0.6)	0.4 ± 0.6
Parous (y/n)	95 (20.4%)	87 (15.6%)	234 (39.0%)	214 (34.4%)
Primary infertility diagnosis				
Male	113 (24.3%)	131 (23.5%)	159 (26.5%)	141 (22.7%)
Tubal	14 (3.0%)	30 (5.4%)	30 (5.0%)	27 (4.3%)
DOR	53 (11.4%)	52 (9.3%)	53 (8.8%)	52 (8.4%)
Ovulatory	48 (10.3%)	79 (14.2%)	69 (11.5%)	70 (11.3%)
Other	237 (51.0%)	266 (47.7%)	289 (48.2%)	332 (53.4%)
Day 3 FSH (IU/mL)	7.2 ± 2.7	7.1 ± 3.6	7.4 ± 4.8	7.2 ± 2.7
AMH (ng/mL)	4.4 ± 4.0	4.8 ± 3.9	5.3 ± 4.9	5.1 ± 5.0
Stimulation protocol ^b				
Antagonist	352 (75.7%)	436 (78.1%)	NA	NA
Low-dose luteal	96 (20.6%)	106 (19.0%)		
Patch and microflare	16 (3.4%)	15 (2.7%)		
Peak E2 (pg/mL) ^b	2210 ± 916	2129 ± 859	NA	NA
Endometrial thickness (mm) ^c	11.2 ± 2.7	10.7 ± 2.8	9.5 ± 2.6	9.5 ± 2.6
Trigger type ^b				
hCG	284 (61.1%)	292 (52.3%)	NA	NA
hCG and Lupron	133 (28.6%)	228 (40.9%)		
N/A (donor or GC)	48 (10.3%)	38 (6.8%)		
ICSI	229 (49.5%)	261 (46.8%)	308 (51.4%)	304 (48.9%)
Good quality embryo transferred	364 (78.3%)	371 (66.5%)	328 (54.7%)	285 (45.8%)
Luteal support				
Vaginal	386 (83.0%)	494 (88.5%)	87 (14.5%)	73 (11.7%)
Intramuscular	76 (16.3%)	61 (10.9%)	492 (82.0%)	529 (85.1%)
Combined/unknown	3 (0.6%)	3 (0.4%)	21 (3.5%)	20 (3.2%)
Expansion grade				
5	28 (6.0%)	51 (9.1%)	88 (14.7%)	136 (21.9%)
6	57 (12.3%)	104 (18.6%)	116 (19.3%)	137 (22.0%)
7	212 (45.6%)	249 (44.6%)	226 (37.7%)	193 (31.0%)
8	168 (36.1%)	154 (27.6%)	170 (28.3%)	156 (25.1%)
ICM grade				
A	167 (35.9%)	179 (32.1%)	134 (22.3%)	108 (17.4%)
B	261 (56.1%)	327 (58.6%)	377 (62.8%)	409 (65.8%)
C	37 (8.0%)	52 (9.3%)	89 (14.7%)	105 (16.9%)

Table 1 (continued)

	Fresh <i>n</i> = 1023		CET <i>n</i> = 1222	
TE grade				
a	199 (42.8%)	193 (34.6%)	136 (22.7%)	102 (16.4%)
b	249 (53.5%)	328 (58.8%)	384 (64.0%)	408 (65.6%)
c	17 (3.7%)	37 (6.6%)	80 (13.3%)	112 (18.0%)

CET, cryopreserved embryo transfer; *BMI*, body mass index; *DOR*, diminished ovarian reserve; *FSH*, follicle-stimulating hormone; *AMH*, anti-Mullerian hormone; *E2*, serum estradiol at the day of hCG administration; *hCG*, human chorionic gonadotropin; *ICSI*, intracytoplasmic sperm injection; *ICM*, inner cell mass; *TE*, trophoctoderm

Values expressed as mean ± SD or *n* (%) as appropriate

^a Frozen cycles only

^b Fresh cycles only

^c Endometrial thickness measured at time of trigger for fresh cycles and time of mapping for frozen cycles

with lower percentages of blastocysts assigned the highest grades for expansion (26.7% vs. 31.5%), ICM (19.8% vs. 33.8%), and TE (19.5% vs. 38.3%) among CET vs. fresh cycles, respectively.

Pregnancy outcomes

Associations between clinical pregnancy rate (CPR) and live birth rate (LBR) for fresh and CET cycles for each of the three morphologic characteristics are shown in Fig. 1. Positive associations were identified for both CPR and LBR with increasing expansion for both cycle types, with significance reached for CPR following fresh transfer, but not CET ($p = 0.001$ vs. $p = 0.221$, respectively) and for LBR in both fresh ($p = 0.005$) and CET ($p = 0.018$) cycles (Table 2). No significant associations were identified for either CPR or LBR with decreasing ICM grade (Table 3). However, both outcomes decreased with decreasing TE quality for both cycle types, with significant associations observed for CPR (fresh, $p = 0.005$; CET, $p < 0.0001$) and LBR (fresh, $p = 0.028$; CET, $p = 0.023$) (Table 4).

Perinatal outcomes

Perinatal outcomes by blastocyst expansion grade are summarized in Table 2. No significant associations between expansion grade and incidence of PTB, SGA, or LGA were identified for either fresh or CET cycles.

Perinatal outcomes by ICM grade are summarized in Table 3. While incidence of PTB was not associated with ICM quality among fresh cycles ($p = 0.388$), there was a significant decrease in rates of PTB with decreasing ICM grade among CET cycles ($p = 0.005$), with mean gestational age at delivery of 38.9 weeks, 39.1 weeks, and 39.3 weeks for grades A, B, and C, respectively. Similarly, mean adjusted birthweight increased with decreasing ICM grade among CET (3420 g, 3555 g, and 3586 g for A, B, and C,

respectively) but not fresh transfers (3373 g, 3354 g, and 3327 g for A, B, and C, respectively); however, no significant associations were observed between ICM grade and incidence of SGA or LGA among CET or fresh cycles.

No significant associations were identified between TE grade and any of the perinatal outcomes evaluated in either fresh or CET cycles (Table 4).

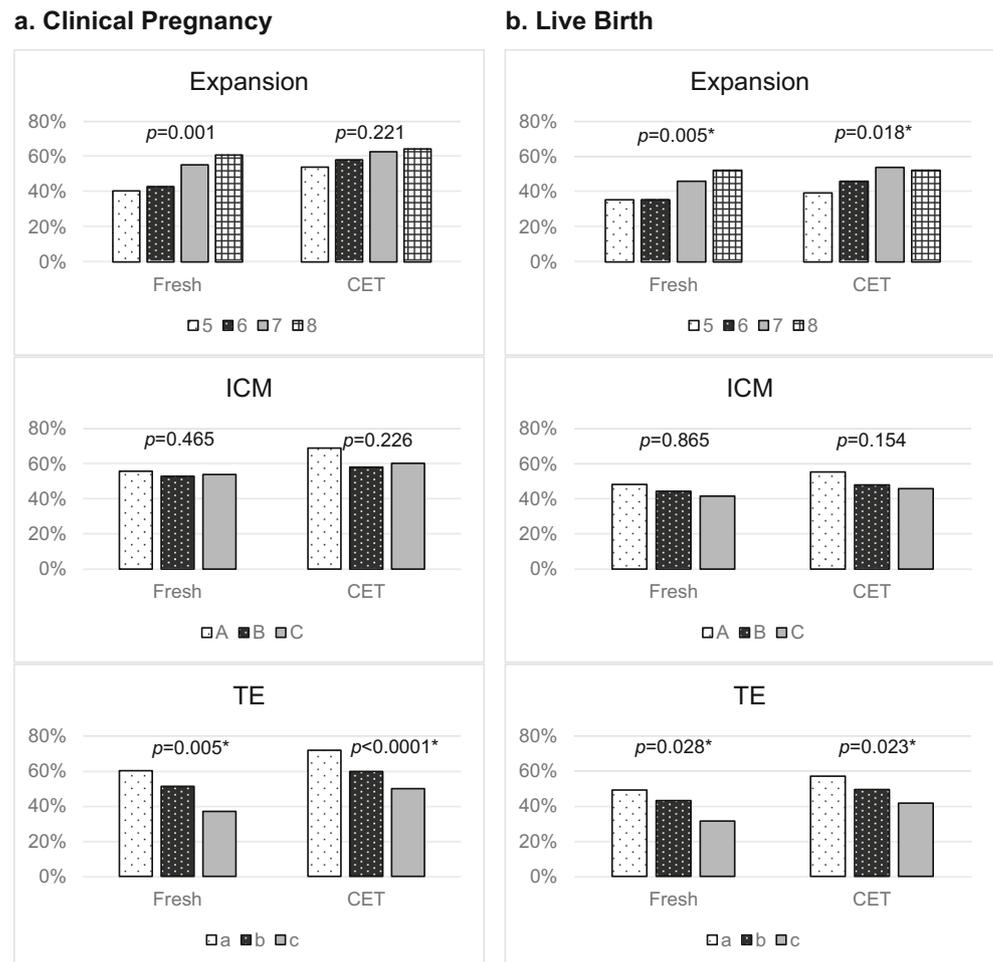
Comparisons of differences between CET and fresh cycles regarding gestational age and adjusted birthweight for each grade within the three morphologic characteristics (expansion, ICM, and TE) are shown in Supplemental Fig. 1. In all cases, the differences were positive in favor of CET and most pronounced for grade 8 blastocysts and blastocysts with ICM and TE grade of C. The overall differences between CET and fresh cycles in gestational age and adjusted birthweight was 0.37 weeks (39.1 vs. 38.7 weeks, CET vs. fresh) and 180 g (3,422 vs. 3242 g, CET vs. fresh), respectively.

Discussion

The aim of this study was to determine whether blastocyst expansion and/or the quality of either the ICM or TE independently is associated with pregnancy and/or perinatal outcomes following SET in fresh or CET cycles. We specifically tested the hypotheses that more advanced expansion and higher grades of TE and ICM would each be associated with an increased likelihood of clinical pregnancy and live birth and a decreased chance of adverse perinatal outcomes. We found that increased expansion and higher trophoctoderm grade were associated with increased probability of clinical pregnancy and live birth. Our hypotheses that increasing expansion and TE and ICM quality would each be further predictive of improved downstream perinatal outcomes were not supported by our data. We observed no associations of perinatal outcomes with expansion and TE quality and, contrary to

Fig. 1 Incidence of **a** clinical pregnancy and **b** live birth by expansion, inner cell mass (ICM), and trophoctoderm (TE) grade in fresh transfers and cryopreserved embryo transfers (CET).

*Statistically significant trend



expectation, found that increasing ICM grade was associated with increased likelihood of PTB among CET cycles.

The association between blastocyst morphology grade, pregnancy, and live birth rate is well supported in the literature [2–10]. The subject of which morphologic characteristic is most predictive of pregnancy outcomes has been less clearly established. Because ICM is fated to become fetal tissue, ICM grade was traditionally thought to be the most significant morphologic characteristic affecting transfer outcomes. However, several recent well-designed studies suggested that TE morphology is of significant importance in predicting pregnancy outcomes [6–10]. The results of the present study support the importance of TE morphology in predicting cycle outcome, with higher TE grade associated with increased likelihood of clinical pregnancy and live birth in both fresh and CET cycles. Given that TE cells give rise to the placenta, it is not surprising that healthy TE cells are required to initiate the complex process of implantation and to support a pregnancy.

While ICM quality was not associated with either clinical pregnancy or live birth in our study, it was found to be an independent predictor of PTB. While the relative importance of the ICM on fetal outcomes makes intuitive sense given the

fate of the ICM to become fetal tissue, our finding that higher ICM grade was paradoxically associated with increased incidence of PTB following CET was unexpected. Blastocysts with an ICM of grade A were nearly twice as likely to result in a preterm delivery as blastocysts with an ICM of grade C. While the mechanism underlying this relationship is not clear, it seems plausible that a more “advanced” ICM at the time of transfer may somehow reflect changes in embryonic and fetal development in utero leading to downstream triggering of earlier delivery. However, there is no evidence to support this theory and, of note, the difference in mean gestational age between ICM grade A and C was only 0.6 weeks. Adjusted birthweight also unexpectedly decreased with higher ICM grade among CET cycles; however, this reduction in birthweight did not lead to a higher incidence of SGA, casting doubt on the clinical significance of these findings.

Interestingly, the association between ICM grade and PTB and the trends in adjusted birthweight were identified only among CET cycles. It has previously been shown that perinatal outcomes including gestational age and birthweight may be improved in CET cycles as compared with fresh cycles [25–28]. Our data further support these findings, with

Table 2 Blastocyst expansion grade and pregnancy and perinatal outcomes

Variable	Cycle type	5 Fresh n = 79 CET n = 224	6 Fresh n = 161 CET n = 253	7 Fresh n = 461 CET n = 419	8 Fresh n = 322 CET n = 326	RR or β (95% CI) 8 (ref) vs. 6	RR or β (95% CI) 8 (ref) vs. 7	p for trend
Clinical pregnancy	Fresh	32 (40.5%)	69 (42.9%)	255 (55.3%)	196 (60.9%)	0.72 (0.54, 0.96)	0.92 (0.82, 1.04)	0.001*
	CET	121 (54.0%)	147 (58.1%)	263 (62.8%)	210 (64.4%)	0.92 (0.79, 1.07)	0.97 (0.90, 1.11)	0.221
Live Birth	Fresh	28 (35.4%)	57 (35.4%)	212 (46.0%)	168 (52.2%)	0.74 (0.53, 1.02)	0.90 (0.78, 1.04)	0.005*
	CET	88 (39.3%)	116 (45.9%)	226 (53.9%)	170 (52.2%)	0.80 (0.66, 0.98)	1.06 (0.93, 1.22)	0.018*
Preterm birth ^{ab} (weeks)	Fresh	3 (10.7%)	8 (14.0%)	25 (11.8%)	26 (15.6%)	0.64 (0.21, 1.95)	0.79 (0.47, 1.32)	0.472
	CET	7 (8.0%)	11 (9.5%)	24 (10.6%)	14 (8.2%)	0.99 (0.40, 2.44)	1.12 (0.51, 2.47)	0.965
Small for gestational age ^{ac}	Fresh	2 (7.4%)	9 (15.8%)	21 (10.0%)	14 (8.4%)	0.73 (0.18, 2.96)	1.60 (0.72, 3.57)	0.818
	CET	6 (8.8%)	7 (6.0%)	15 (6.7%)	9 (5.3%)	1.45 (0.52, 4.08)	1.19 (0.47, 3.00)	0.519
Large for gestational age ^{ac}	Fresh	4 (14.8%)	8 (14.0%)	15 (7.1%)	14 (8.4%)	Did not converge	Did not converge	–
	CET	13 (14.8%)	24 (20.7%)	32 (14.2%)	24 (14.1%)	1.00 (0.54, 1.86)	1.41 (0.85, 2.35)	0.532

Ref, referent variable; RR, relative risk; β , beta coefficient; CI, confidence interval

Values expressed as mean \pm SD or n (%) as appropriate. All analyses adjusted for maternal age and BMI

^a Among live births

^b Preterm birth defined as delivery before 37 weeks' gestation

^c Small for gestational age and large for gestational age = adjusted birthweight \leq 10th percentile and \geq 90th percentile, respectively

*Statistically significant result

increased gestational age and birthweight noted for CET as compared with fresh cycles across all grades for all three morphologic characteristics, with the greatest differences observed between CET and fresh blastocysts with an expansion grade of 8 or with ICM or TE grade of C (Supplemental Fig. 1). Possible explanations include epigenetic modifications induced by the process of cryopreservation, disturbances in the bidirectional communication between the embryo and uterine epithelial cells, and differences in the hormonal environment of the uterus as a result of recent ovarian stimulation in fresh cycles [29]. While the mechanism has not been firmly established, our results contribute to a growing body of research suggesting that fresh and CET cycles may be characterized by different determinants of gestational age and birthweight.

Overall, our findings are in contrast to those from the limited number of previous studies relating to the association between individual morphologic characteristics and perinatal outcomes. A 2015 study from Licciardi et al. analyzing 224 live births resulting from fresh and slow-frozen and vitrified blastocyst-stage SET found that an ICM grade of A among fresh transfers was associated with higher birthweight as compared with an ICM grade of B; however, no significant difference was noted between an ICM grade of A vs. a grade of C, which the authors attributed to the very low number of embryos with the lowest ICM grade with only 5 live births resulting from such embryos. No effect was identified between ICM grade and birthweight in CET cycles or between TE grade and birthweight in fresh or CET cycles [15]. A 2016 study from Ebner et al. involving 225 fresh and cryopreserved blastocyst-stage SET found that among 139 live births, none of the three characteristics were associated with birthweight or gestational age; however, this study omitted embryos with the lowest TE and ICM grades given very low numbers of live births within in these groups [16]. Finally, a 2017 study from Miyazaki et al. found that, among 828 live births following cryopreserved blastocyst-stage SET, there was no difference in birth length, birthweight, or congenital anomalies based on ICM or TE grade; however, only 2 of the 828 embryos in this study (0.24%) received an ICM grade of C [17]. The relatively small numbers of live births analyzed in these prior studies is a clear limitation. These small numbers are particularly limiting when the proportion of embryos with the lowest morphologic grades is so low, e.g. with the number of ICM grade C blastocysts in each of these studies ranging from 2 to 7 compared with 129 blastocysts in the present study. Overall, our study included 4-fold the number of live births compared with that of Licciardi et al. and 7-fold compared with that of Ebner et al. Further, differences in the relative distribution of blastocysts of each morphologic grade among studies point to some subjectivity in assigning morphologic grade or varying practices in embryo culture and selection which may have further contributed to the discrepant results.

Table 3 Inner cell mass grade and pregnancy and perinatal outcomes

Variable	Cycle type	Inner cell mass grade			RR or β	RR or β	<i>p</i> for trend
		A	B	C	(95% CI) A (ref) vs. B	(95% CI) A (ref) vs. C	
		Fresh <i>n</i> = 346 CET <i>n</i> = 242	Fresh <i>n</i> = 588 CET <i>n</i> = 786	Fresh <i>n</i> = 89 CET <i>n</i> = 194			
Clinical pregnancy	Fresh	193 (55.8%)	311 (52.9%)	48 (53.9%)	1.03 (0.91, 1.16)	1.08 (0.87, 1.33)	0.465
	CET	176 (69.0%)	457 (58.1%)	117 (60.3%)	0.92 (0.82, 1.02)	0.92 (0.80, 1.07)	0.226
Live birth	Fresh	167 (48.3%)	261 (44.4%)	37 (41.6%)	1.00 (0.86, 1.15)	0.97 (0.86, 1.15)	0.865
	CET	134 (55.4%)	377 (48.0%)	89 (45.9%)	0.96 (0.84, 1.11)	0.88 (0.73, 1.07)	0.154
Preterm birth ^{a,b}	Fresh	26 (15.6%)	31 (11.9%)	5 (13.5%)	0.74 (0.44, 1.23)	0.87 (0.35, 2.15)	0.388
	CET	21 (15.7%)	30 (8.0%)	5 (5.6%)	0.46 (0.26, 0.81)	0.32 (0.12, 0.83)	0.005*
Small for gestational age ^{a,c}	Fresh	15 (9.0%)	25 (9.7%)	6 (16.2%)	0.96 (0.51, 1.81)	1.40 (0.60, 3.28)	0.630
	CET	9 (6.7%)	22 (5.6%)	6 (6.7%)	0.93 (0.42, 2.08)	1.00 (0.36, 2.76)	0.967
Large for gestational age ^{a,c}	Fresh	14 (8.4%)	24 (9.3%)	3 (8.1%)	Did not converge	Did not converge	–
	CET	16 (11.9%)	61 (16.2%)	16 (18.0%)	1.26 (0.74, 2.16)	1.45 (0.75, 2.79)	0.243

Ref, referent variable; *RR*, relative risk; β , beta coefficient; *CI*, confidence interval

Values expressed as mean \pm SD or *n* (%) as appropriate. All analyses adjusted for maternal age and BMI

^a Among live births

^b Preterm birth defined as delivery before 37 weeks' gestation

^c Small for gestational age and large for gestational age = adjusted birthweight \leq 10th percentile and \geq 90th percentile, respectively

*Statistically significant result

Notably, this study included only unbiopsied embryo transfers, and therefore cannot exclude the effect of embryo aneuploidy on pregnancy and perinatal outcomes. A recent study of biopsy-proven euploid blastocyst CET cycles found that TE

and ICM grade but not degree of blastocyst expansion were associated with clinical pregnancy and live birth [30]. While these findings further support the importance of TE morphology in predicting cycle outcomes, they are in contrast to our

Table 4 Trophoctoderm grade and pregnancy and perinatal outcomes

Variable	Cycle Type	Trophoctoderm grade			RR or β	RR or β	<i>p</i> for trend
		a	b	c	(95% CI) a (ref) vs. b	(95% CI) a (ref) vs. c	
		Fresh <i>n</i> = 392 CET <i>n</i> = 238	Fresh <i>n</i> = 577 CET <i>n</i> = 792	Fresh <i>n</i> = 54 CET <i>n</i> = 192			
Clinical pregnancy	Fresh	236 (60.2%)	296 (51.3%)	20 (37.0%)	0.88 (0.79, 0.99)	0.69 (0.48, 0.99)	0.005*
	CET	171 (71.9%)	474 (59.8%)	96 (50.0%)	0.85 (0.77, 0.95)	0.73 (0.61, 0.87)	< 0.0001*
Live birth	Fresh	199 (49.2%)	249 (43.2%)	17 (31.5%)	0.89 (0.78, 1.02)	0.70 (0.46, 1.06)	0.028*
	CET	136 (57.1%)	384 (49.5%)	80 (41.7%)	0.90 (0.78, 1.03)	0.90 (0.78, 1.03)	0.023*
Preterm birth ^{a,b}	Fresh	29 (14.6%)	29 (11.7%)	4 (23.5%)	0.83 (0.51, 1.33)	2.14 (0.82, 5.57)	0.936
	CET	13 (9.6%)	36 (9.4%)	7 (8.8%)	1.10 (0.58, 2.11)	1.19 (0.46, 3.07)	0.747
Small for gestational age ^{a,c}	Fresh	13 (6.6%)	31 (12.6%)	2 (11.8%)	1.80 (0.96, 3.36)	1.80 (0.45, 7.12)	0.054
	CET	8 (5.9%)	26 (6.8%)	3 (3.8%)	1.18 (0.52, 2.70)	0.66 (0.18, 2.45)	0.644
Large for gestational age ^{a,c}	Fresh	20 (10.1%)	21 (8.5%)	0 (0.0%)	Did not converge	Did not converge	–
	CET	19 (14.1%)	60 (15.6%)	14 (17.5%)	1.00 (0.61, 1.63)	1.11 (0.57, 2.17)	0.720

Ref, referent variable; *RR*, relative risk; β , beta coefficient; *CI*, confidence interval

Values expressed as mean \pm SD or *n* (%) as appropriate. All analyses adjusted for maternal age and BMI

^a Among live births

^b Preterm birth defined as delivery before 37 weeks' gestation

^c Small for gestational age and large for gestational age = adjusted birthweight \leq 10th percentile and \geq 90th percentile, respectively

*Statistically significant result

findings regarding ICM and blastocyst expansion and raise concern regarding the applicability of our results to known euploid embryo transfers. While prior studies have identified associations between blastocyst morphology and ploidy status [31, 32], further studies are needed to clarify how the predictive value of morphology may vary between euploid and aneuploid embryos. For now, caution should be exercised to avoid overgeneralization of our results.

Further limitations of this study include its retrospective design and the relatively small numbers of embryos with low ICM and TE grades, although these numbers far exceed those from previous studies in the literature. Given the widespread practice of selectively transferring embryos with higher morphologic grades, inclusion of larger numbers of embryos with the lowest morphologic grades would require either a randomized controlled trial (which is unlikely to occur given the evidence supporting the predictive value of morphology on pregnancy and live birth) or a vastly larger number of cycles. It is therefore possible that our study was underpowered, particularly as no a priori power analysis was conducted.

Despite these limitations, this study had several key strengths. To our knowledge, it is the largest study to evaluate the association between individual morphologic characteristics and perinatal outcomes. The single-center design ensured homogenous embryo grading and selection practices as well as embryo culture processes. Inclusion of both fresh and CET transfers allowed for comparison of the associations identified between the two types of cycles, identifying that trends may vary between fresh and CET cycles. Finally, the use of blastocyst fresh and cryopreserved embryo transfers renders our results highly relevant to current practice.

In conclusion, our findings contribute to a growing body of literature supporting the predictive value of blastocyst morphology on pregnancy and live birth and demonstrate interesting associations between ICM grade, birthweight, and preterm birth among CET cycles. Regarding current clinical practice, our results should reassure clinicians and patients alike that pregnancies arising from fresh or cryopreserved SET of less expanded blastocysts or blastocysts of lower ICM or TE quality do not appear more likely to result in adverse perinatal outcomes.

Compliance with ethical standards

This study was approved by the Partners Human Research Committee at the Brigham and Women's Hospital (Protocol #2018P000317). All data was retrieved from standard records collected during clinical care, thus informed consent was not required.

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

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