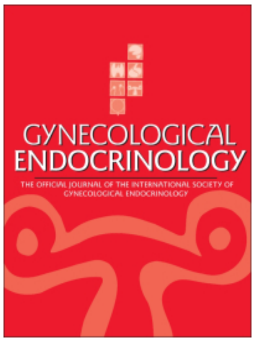

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Implantation in single euploid embryo transfers: the role of blastocyst expansion grade and early serum β-hCG levels

Arthur C. Arcaz[a], Carlos Hernandez-Nieto[b], Joseph A. Lee[b], Tanmoy Mukherjee[a,b] and Alan B. Copperman[a,b]

[a]Obstetrics, Gynecology and Reproductive Science, Icahn School of Medicine at Mount Sinai, New York, New York, USA; [b]Reproductive Medicine Associates of New York, New York, New York, USA

ABSTRACT

Objective: Is there an association between blastocyst expansion grade and early serum β-hCG levels in pregnancies after single euploid embryo transfer?
Methods: This retrospective cohort study included 8394 SEETs performed at a single center. Blastocysts were categorized as CHBs ($n = 1677$) or PHBs ($n = 4612$) based on their expansion grade. Serum β-hCG levels were measured on days 9 and 11 post-transfer. A multivariate logistic regression model adjusted for confounders, including maternal age, ovarian reserve, embryo morphology, year of treatment, and day of biopsy.
Results: On day 9 post-transfer, the median β-hCG level was significantly lower in CHBs (118 mIU/mL, IQR = 129.2) compared to PHBs (133 mIU/mL, IQR = 129.8; $p < 0.0001$). The difference persisted on day 11 post-transfer (CHBs: 290.2 mIU/mL, IQR = 357 vs. PHBs: 327.3 mIU/mL, IQR 358.6; $p = 0.018$). In multivariate regression, CHB status remained significantly associated with lower β-hCG on day 9 post-transfer (aOR = 0.998, 95% CI: 0.997–0.999), but not on day 11 post-transfer (aOR = 0.999, 95% CI: 0.999–1.0002).
Conclusions: Completely hatched blastocysts (CHBs) with an expansion grade 6 were associated with lower early serum β-hCG levels compared to partially hatched blastocysts (PHBs) with expansion grades 4 and 5. Incorporating expansion grade into prediction models could enhance decision support tools for IVF patients.

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Blastocyst; embryo transfer; human chorionic gonadotropin; in vitro fertilization; pregnancy outcome

Introduction

Embryo implantation begins with the loss of the zona pellucida (ZP), a process known as hatching, which occurs 1–3 d after the embryo enters the uterine cavity [1]. Human chorionic gonadotropin (hCG), produced by the implanting trophoblast, serves as an early and reliable marker of implantation. Serum β-hCG measurements after a single embryo transfer (SET) remain the earliest predictive indicator of pregnancy outcomes in IVF cycles [2]. Specifically, parameters such as β-hCG 'doubling' rates and reaching specific thresholds by day 15 post-transfer are robust predictors of live birth [3].

Today, the selection of embryos for transfer relies on genomic assessment, however morphological assessment of blastocysts still holds value, with grading systems like Gardner's scale [4] playing a complementary role. In addition to assessing the quality of the inner cell mass (ICM) and trophectoderm (TE), this scale also determines an expansion grade, which reflects the embryo's progress in hatching out of the ZP and has been associated with implantation potential [5]. Assisted hatching before transfer has also demonstrated increased implantation rates, especially in poor quality blastocysts [5,6]. These differences suggest that the removal of the zona pellucida (ZP) during hatching could influence early serum hCG levels. However, this relationship between blastocyst expansion grade and early β-hCG levels in maternal serum has not been well studied.

CONTACT Arthur C. Arcaz arthur.arcaz@mountsinai.org Obstetrics, Gynecology and Reproductive Science, Icahn School of Medicine at Mount Sinai, 1 Gustave L. Levy Pl, New York, New York, 10029, USA
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Prior studies have speculated that assisted hatching may accelerate embryo-endometrium contact, reflected in earlier detectable via rise of β-hCG, though findings have been inconsistent and limited by small sample sizes [7,8]. More recently Girard et al. [9] showed that β-hCG kinetics – calculated through serial measurements over time to reduce variability – differ across blastocyst expansion grades, with higher kinetic rates observed in expansion grade 3 compared to expansion grades 2 or 4. These studies have provided preliminary insights into the relationship between expansion grade and implantation dynamics, though questions remain, as none included fully hatched blastocysts in cohorts restricted to euploid embryos.

In response, the present study compared early serum β-hCG kinetics in singleton pregnancies from the transfer of fully hatched (expansion grade 6) versus unhatched (expansion grades 4 or 5) euploid blastocysts from IVF cycles with Preimplantation Genetic Testing for Aneuploidy (PGT-A). By measuring β-hCG levels at fixed post-transfer intervals and controlling for potential confounders, this study aims to determine whether hatching status at the time of embryo transfer correlates with differences in early β-hCG kinetics.

Materials and methods

Methods

A retrospective cohort analysis was performed at a single, private-academic ART center and included couples who underwent an IVF/PGT-A cycle with a single euploid blastocyst transfer (SEET) within the study period (September 2016 to March 2024). All patients underwent controlled ovarian hyperstimulation (COH), ICSI, extended embryo culture, and TE biopsy. As part of routine laboratory practice, all embryos underwent assisted hatching on day 3 of development to facilitate subsequent biopsy. Trophectoderm biopsy was performed on day 5, 6, or 7 depending on the embryo's developmental stage. Following biopsy, embryos were vitrified after a variable interval dictated by laboratory workload, with vitrification occurring within 45 min on average (range 30 min to 2 h). PGT-A analyzes were performed with next-generation sequencing technology. All ovarian stimulation protocols and laboratory methods used in the study had been previously described [10].

Blastocysts grading was performed post-thaw, immediately prior to embryo transfer, using a center-specific scoring system (modified Gardner scale, see Supplementary Table 1) previously described for blastocysts intended to undergo TE biopsy for PGT [11,12]. Grading was performed by senior embryologists at the fertility center. As part of the center's internal quality assurance program, embryologists participate in routine calibration sessions and inter-observer reliability exercises to maintain grading consistency.

For embryo transfer, all cases underwent synthetic endometrial preparation as previously described [13]. Thawing and transfer were performed on the sixth day of progesterone supplementation, regardless of the embryo's developmental day at cryopreservation. Euploid embryos with the highest morphology grade were selected for transfer. In cases involving gender selection for family balancing, the highest graded embryo of the preferred genetic sex was transferred. Day 5 biopsied embryos were preferentially selected over day 6 when available and of the same grade. Among embryos biopsied on the same day of development, ICM grade was prioritized in embryo selection, followed by expansion grade, and then TE grade. This hierarchy reflects our center's protocol and is supported by previous findings that ICM morphology is the strongest predictor of implantation potential in euploid embryos as previously described [13].

Serum β-hCG levels were measured for all cases on days 9 and 11 post-transfer using electrochemiluminescence immunoassay (Immulite 2000 and Cobas e–601). Blood collection was performed within a standardized window (7:00 a.m. to 10:00 a.m.) on both days to minimize diurnal variation and pre-analytical variability. Samples were targeted to be drawn 48 h apart. Both assay platforms are validated and demonstrate consistent performance across the measured range.

Cohorts were categorized based on blastocyst expansion grade: Completely Hatched Blastocysts (CHB) with an expansion grade 6, and a control group of Partially Hatched Blastocysts (PHB) with expansion grades 4 or 5. Only cases with a positive pregnancy test – defined as a β-hCG value of ≥2.5 mIU/mL measured 9 d post-transfer – were included. Exclusion criteria encompassed cases with β-hCG levels measured on different days, incomplete information, multiple TE biopsies, multiple thaw/freeze procedures, use of donor oocytes, testicular sperm extraction, known chromosomal rearrangements, monozygotic splitting, ectopic pregnancies, or pregnancies of unknown location. Ectopic pregnancies were excluded due to diagnostic ambiguity in distinguishing them from pregnancies of unknown location, and because the small sample size precluded meaningful analysis.

Outcomes

The primary outcome was the association between the first β-hCG measurement (mIU/mL) at 9 d post-transfer and blastocyst expansion grade, adjusting for important co-variables. Both absolute β-hCG values and the relative changes between measurements on days 9 and 11 post-transfer (β-hCG kinetics) were evaluated.

Secondary outcomes included clinical pregnancy rate (CPR): the proportion of patients with ultrasound-confirmed fetal cardiac activity; biochemical pregnancy loss rate (BPL): pregnancy loss occurring after the presence of a positive pregnancy test followed by a decrease or lack of increase of β-hCG serum levels in serial measurements 48 h after the first measurement and/or without detection of a gestational sac visualized by vaginal ultrasound at the fifth week of pregnancy; clinical pregnancy loss rate (CPL): pregnancy loss occurring after the presence of a confirmed gestational sac; and live birth rate (LBR): sustained pregnancy after detected fetal heartbeat on a vaginal ultrasound and/or complete delivery of a product of fertilization after ≥22 completed weeks of gestational age, which breathes or shows evidence of life [14].

Statistical analysis

Statistical analyzes were performed using SAS version 9.4 (SAS Institute Inc. Cary, NC, USA). Demographic, COH, and embryological data were recorded for all participants. Descriptive and unadjusted comparative analyzes were performed using ANOVA, Kruskal–Wallis, Fisher's exact test, and chi-squared tests where appropriate. Point biserial correlation was employed to evaluate the relationship between β-hCG levels and expansion grade. A multivariate logistic regression analysis was performed and fitted with generalized estimating equations (GEE) to account for patients who underwent multiple COH or FET cycles. Adjusted odds ratios (aOR) with 95% confidence intervals (CI) were calculated. All variables that showed significance on the unadjusted analysis and/or variables that were thought to be clinically relevant were encompassed and adjusted for as covariates in the models. Day of embryo biopsy (day 5 vs. 6) was included as a covariate in all multivariate models to account for potential differences in embryo developmental timing. Previous data from our center have shown that days 5 and 6 euploid embryos have comparable clinical outcomes, while day 7 embryos are associated with significantly lower implantation and live birth rates [12]. In addition, adjustment for year of treatment was included in all multivariate models to account for changes in practices. All p-values were two sided with a statistical significance level set at $p < 0.05$. A power analysis determined that a sample size of 329 SEETs per group was needed to detect a 10% difference in β-hCG levels with 80% power and an alpha of 0.05.

Ethics approval

This retrospective analysis was approved by an academic Institutional Review Board (HS #: STUDY-18-00441). Patient information was de-identified before data analysis.

Results

A total of 8394 SEETs were eligible for analysis. Of these, a total of 6289 had a positive pregnancy test and β-hCG measurements on days 9 and 11 after embryo transfer and were included in the final analysis. The final cohort included 4612 cases in the PHB group (expansion grade 4 or 5) and 1677 cases in the CHB group (expansion grade 6).

Baseline characteristics

Univariate analysis findings are shown in Table 1. Significant differences between the PHB and CHB groups were observed in oocyte age, age at embryo transfer, AMH, baseline antral follicular count (BAFC) and the number of prior embryo transfers per patient. There were no significant differences in body mass index (BMI), peak serum estradiol prior to transfer, peak serum progesterone on the day of intramuscular progesterone initiation, or endometrial thickness at embryo transfer.

β-hCG levels and kinetics

On day 9 after embryo transfer (ET), the median β-hCG level in the CHB group was 118 mIU/mL (IQR = 129.2) compared with 133 mIU/mL (IQR = 129.8) in the PHB group ($p < 0.0001$). On day 11 post-transfer, the median β-hCG level in the CHB group was 290.2 mIU/mL (IQR = 357) compared with 327.3 mIU/mL (IQR = 358.6) in the PHB group ($p = 0.018$) ($p =< 0.018$).

The proportion of cases achieving ≥100% increase in β-hCG levels from day 9 to 11 post-transfer was comparable between CHB (76.7%) and PHB (77.1%) ($p = 0.91$). These comparisons are shown in Tables 1 and 2.

A point-biserial correlation analysis showed a significant association between CHB and lower β-hCG levels on day 9 (Rho = −0.06; $p < 0.0001$) and a weaker, but still significant, association on day 11 (Rho = −0.04; $p = 0.006$).

Pregnancy outcomes

The clinical pregnancy loss rate was significantly higher in the CHB group compared with the PHB group (14.5% vs. 12.5%; $p = 0.03$; Table 2). Clinical pregnancy rate (CPR) and biochemical pregnancy loss rates

Table 1. Baseline characteristics and early β-hCG levels of patients undergoing SEET, stratified by blastocyst hatching status.

	Completely hatched (CHB) $n = 1677$		Partially hatched (PHB) $n = 4612$		
	Median	IQR	Median	IQR	*P*-value
Oocyte age (years)	35.7	5.5	35.6	5.5	0.003
Age at ET (years)	36.6	5.5	36.3	5.6	0.0005
Gravidity (mean, SD)	1.37	1.2	1.45	1.28	0.08
Parity (mean, SD)	0.68	0.75	0.69	0.78	0.73
BMI (kg/m^2)	23.0	5.5	23.2	5.4	0.43
Year of treatment (mean, SD)	2019	2.5	2018	2.7	<0.0001
Day of biopsy (count, %)					<0.0001
5	3074	66.7%	644	38.4%	
6	1448	31.4%	947	56.5%	
7	90	2.0%	86	5.1%	
AMH (ng/ml)	2.3	2.9		3.1	<0.0001
			2.6		
BAFC	12.0	9.0	12.0	11.0	0.01
Peak serum Estradiol pre-ET (pg/ml)	277.5	189.5	268.7	185.6	0.35
Peak serum Progesterone before conversion (ng/ml)	0.3	0.2	0.3	0.2	0.21
Endometrial Thickness at ET (mm)	9.5	3.0	9.5	3.0	0.43
Number of prior ETs	0.0	1.0	0.0	1.0	0.002
β-hCG 9 d post-ET (mIU/ml)	118	129.2	133.4	129.8	<0.0001
β-hCG 11 d post-ET (mIU/ml)	290.2	357	327.3	358.6	0.018

SD: Standard Deviation; BMI: Body Mass Index; AMH: Anti-Mullerian Hormone; BAFC: Baseline Antral Follicular Count; ET: Embryo Transfer. All continuous variables are presented as median (IQR) unless otherwise specified; categorical variables (day of biopsy) are shown as *n* (%).

Table 2. Clinical pregnancy outcomes and β-hCG kinetics, stratified by blastocyst hatching status.

	Completely hatched (CHB) $n = 1677$		Partially hatched (PHB) $n = 4612$		
	N	Proportion	N	Proportion	*P* value
≥100% β-hCG increase from days 9 to 11	1287	76.7	3556	77.1	0.91
Clinical pregnancy rate	1398	83.36	3859	83.67	0.76
Live birth rate	1154	68.81	3282	71.6	0.07
Clinical pregnancy loss	244	14.55	577	12.51	0.03
Biochemical pregnancy loss	279	16.64	753	16.33	0.76
Twin pregnancy rate	31	1.85	110	2.39	0.2

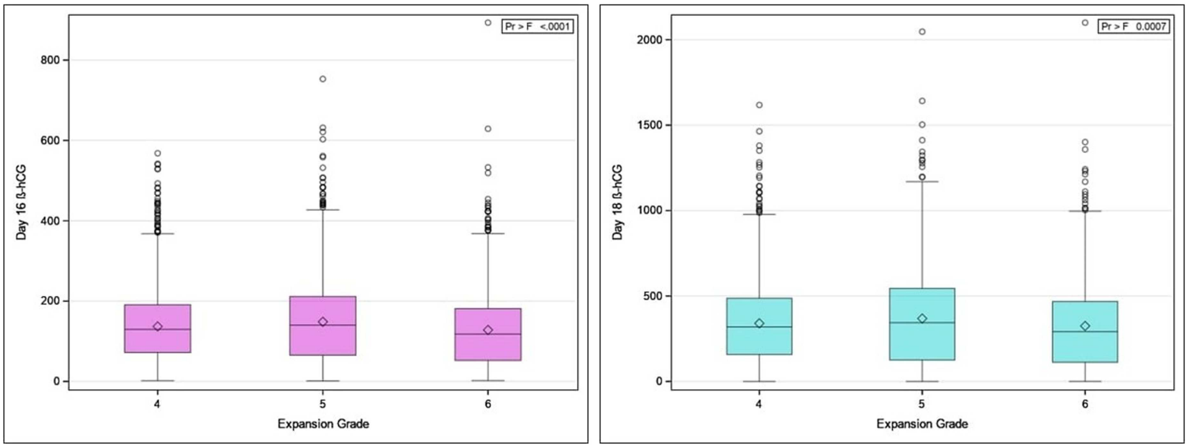

Figure 1. Median serum β-hCG levels stratified by blastocyst expansion grades. (a) Median β-hCG levels measured 9 d post-embryo transfer for blastocyst expansion grades 4, 5, and 6. (b) Median β-hCG levels measured 11 d post-embryo transfer for blastocyst expansion grades 4, 5, and 6. Significant differences in β-hCG levels were observed among the groups at both time points.

were comparable between CHB and PHB groups ($p = 0.76$ and $p = 0.76$, respectively), as was the live birth rate ($p = 0.07$; Table 2).

Multivariate regression analysis

After adjusting for oocyte age, age at ET, BMI, AMH, year of treatment, day of embryo biopsy, and ICM and TE grades, the regression analysis revealed a significant association between CHB status and lower β-hCG levels on day 9 post-ET (aOR 0.998, 95% CI: 0.997–0.999). This association was not observed for β-hCG levels on day 11 post-ET (aOR 0.999, 95% CI: 0.999–1.0002).

Subgroup analysis by expansion grade

When analyzing expansion grades separately (Exp 4, $n = 2728$; Exp 5, $n = 1884$; Exp 6, $n = 1677$), significant differences emerged in median β-hCG levels at day 9 post-ET ($p < 0.0001$) and day 11 post-ET ($p = 0.0008$). Specifically, on day 9 post-ET, median β-hCG levels were 129.7 mIU/mL (IQR = 119.01) for Exp 4, 140.1 mIU/mL (IQR = 145.96) for Exp 5, and 118 mIU/mL (IQR = 129.2) for Exp 6 (Figure 1a). On day 11 post-ET, median β-hCG levels were 318.5 mIU/mL (IQR = 329.6) for Exp 4, 343.3 mIU/mL (IQR = 420) for Exp 5, and 290.2 mIU/mL (IQR = 357) for Exp 6 (Figure 1b). Finally, a correlation analysis confirmed a significant association between expansion grade 6 embryos and lower β-hCG values (Rho = −0.02, $p = 0.02$).

Discussion

This large cohort analysis found that completely hatched blastocysts (CHB, expansion grade of 6) were associated with lower early serum β-hCG levels compared to partially hatched blastocysts (PHB, expansion grades of 4 and 5). However, the proportion of cases achieving a ‘doubling’ of β-hCG within 48 h was similar between the two groups, suggesting that hatching status is not associated with the dynamics of serum β-hCG production in vivo.

Unlike previous studies that focused on implantation rates across hatching status, this study focuses on early β-hCG levels as the primary outcome. Furthermore, prior works have addressed the relationship between β-hCG levels and TE and ICM morphology, including a recent study demonstrating a correlation between TE grade and initial β-hCG levels in SEETs [5,9,11]. In the present study, these morphological parameters were included as covariates to isolate the effect of expansion grade on β-hCG.

β-hCG production is an early indicator of embryo-endometrial receptivity as its production increases after implantation by trophoblast [15]. The appearance of β-hCG in serum has been used to extrapolate implantation timing [8,16]. Thus, one might expect that fully hatched embryos would achieve higher β-hCG levels by day 9 post-transfer due to a possible advantage in early contact with the endometrium. However, the results in this study suggest that blastocyst expansion grade does not uniformly correlate with early trophoblastic activity as reflected by serum β-hCG.

Several biological factors may explain this observation. Successful implantation depends on the quality of the hatched blastocyst, the endometrial receptivity and the interaction between the two [17]. Having hatched in vitro, CHBs may experience a disruption in the natural temporal synchronization with the endometrium, potentially impairing early trophoblast invasion or hormonal signaling [1,18-20]. PHBs may complete hatching in vivo after transfer, potentially synchronizing their developmental stage with the receptive endometrium. In addition, the absence of the ZP could render CHBs more susceptible to stress during trophectoderm biopsy and vitrification/warming, possibly compromising TE integrity. It is also conceivable that the lack of a zona barrier alters subsequent TE differentiation trajectories, independent of embryo–endometrial interactions.

The loss of the ZP is also known to influence the isoform of hCG produced. CHBs preferentially produce hCG-H, which is required for successful implantation [21,22]. While the hCG assay in this study measures different hCG isoforms, including hCH-H, the relative hCG isoform distribution may be more important than the actual overall hCG level. Future studies may benefit from examining the clinical utility of evaluating hCG and hCG-H separately in maternal serum. Concurrently, the loss of the ZP could expose the CHBs to greater oxidative stress and inflammatory cytokines pre-transfer in culture media or post-transfer in the uterine environment, potentially disrupting trophoblast function in the very early stages [23-25].

In terms of secondary pregnancy outcomes, this study demonstrated differences in the clinical pregnancy loss rate, which was higher in the CHB group as compared to the PHB group. While this finding could reflect underlying biological differences in trophoblast development or endometrial receptivity timing, it is also possible that it represents a subtle vulnerability that becomes evident later in pregnancy. For instance, the early stage at which CHBs emerge from the zona pellucida might influence trophoblast differentiation, implantation site selection, or the robustness of the maternal-embryo interaction. Alternately, the slight disadvantage could be related to other confounding variables not fully captured in the study model, despite adjustment for key factors such as oocyte age, maternal age, ovarian reserve indicators, and other embryo morphological parameters, like ICM and TE. While low serum β-hCG levels and suboptimal rise patterns have been previously associated with adverse pregnancy outcomes, our study was not designed or powered to stratify miscarriage rates based on β-hCG kinetics.

There are limitations to this study. First, the retrospective design and single-center setting may restrict the generalizability of the findings. Additionally, all embryos analyzed in this study underwent TE biopsy, which may not fully replicate the dynamics observed in natural pregnancies or embryos that have not undergone biopsy [26]. Another limitation of this study is that embryos were classified as completely or partially hatched based on morphology at the time of freezing, and we could not uniformly determine whether hatching occurred prior to biopsy or after thawing. While we observed differences in clinical pregnancy loss between CHBs and PHBs, this study was not designed to investigate independent predictors of CPL, and caution should be used when interpreting secondary outcomes. Finally, while all hCG measurements were performed using consistent assays at the study center, differences in hCG measuring assays used across other laboratories may limit the broader applicability of these findings.

In conclusion, CHBs were shown to have slightly lower early serum β-hCG levels and a higher rate of early pregnancy loss compared to partially hatched blastocysts (PHBs), despite comparable frequencies of achieving a 'doubling' of β-hCG within 48 h. These findings suggest that the complete loss of the zona pellucida prior to transfer may alter embryo-endometrial interactions or possibly expose CHBs to oxidative stress during culture or after transfer, impairing trophoblast function, and reducing implantation potential. While the associations were statistically significant, the absolute effect sizes were modest, and the clinical utility of expansion grade as an isolated predictor of outcome appears limited.

Future studies should focus on measuring and evaluating hCG isoforms separately to better understand their specific contributions to implantation and early pregnancy outcomes. Additionally, investigating the molecular and biochemical pathways affected by the loss of the zona pellucida could further illuminate the nuanced relationship between blastocyst expansion grade and early trophoblast activity. Such advancements could optimize embryo selection criteria and ultimately improve outcomes in assisted reproductive technology.

Author contributions

CRediT: ACA: formal analysis, investigation, data curation, writing – original draft. CH-N: conceptualization, methodology, formal analysis, investigation, data curation, writing – review and editing, supervision. JAL: validation, resources, writing – review and editing, project administration. TM: conceptualization, writing – review and editing. ABC: conceptualization, writing – review and editing, supervision.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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ORCID

Carlos Hernandez-Nieto 0000-0002-6703-1341

Data availability statement

The data that support the findings of this study are available from the corresponding author, ACA, upon reasonable request.

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