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ORIGINAL ARTICLE

Biological relevance of trophectoderm morphology: initial β-hCG measurements correlate with trophectoderm grading on euploid frozen embryo transfers

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Abstract

Objective To analyze the correlation between TE grading and initial β-hCG serum level after single euploid embryo transfer. Secondarily, to explore the association between TE grading with subsequent IVF outcomes.

Design Retrospective cohort analysis.

Setting Single, academic, private infertility and assisted reproductive care institute.

Patients or other participants Infertility patients who underwent a single euploid embryo transfer that resulted in a positive pregnancy test.

Intervention(s) β-hCG measurements.

Main outcome measure(s) Correlation between TE grade with first β-hCG measurement. Second outcome measurements included ongoing pregnancy, biochemical pregnancy loss, and clinical pregnancy loss rates.

Results 2,798 cases were analyzed. A significant difference in initial β-hCG measurement among groups (TE A: median 143.4 mIU/mL IQR 79.2–211.2; TE B: 119 mIU/mL IQR 57.1–177.8; TE C: 82.4 mIU/mL IQR 36.3–136.4, $p \leq 0.0001$) was observed. There was a significant correlation found between the TE grade and β-hCG measurements ($p \leq 0.0001$, $r^2 = 0.10$). TE grade was not associated with higher odds of biochemical pregnancy loss (TE A vs. TE B: aOR 1.01 CI95% 0.97–1.05; TE A vs. TE C: aOR 1.03 CI95% 0.98–1.08), or higher odds of clinical pregnancy loss (TE A vs. TE B: aOR 1.02 CI95% 0.98–1.05; TE A vs. TE C: aOR 1.03 CI95% 0.98–1.07).

Conclusions In patients with euploid embryos, TE grade correlates with the first pregnancy test measurement of β-hCG. We propose this finding helps to appoint a relevant link between morphology assessment and early embryo development in vivo.

Keywords Human chorionic gonadotropin · Embryo implantation · Trophectoderm · Blastocyst · In vitro fertilization · Pregnancy test

Introduction

Beta human chorionic gonadotrophin (β-hCG) is a hormone sub-unit that contains 145 amino acids and is secreted by placental synciotrophoblast tissue during early pregnancy [1]. After assisted reproduction technology (ART) treatment, β-hCG is detectable in maternal serum as early as 8 days after ovulation; 8–11 days after oocyte retrieval; or 6–8 days after fertilization [2]. This initial detection of β-hCG in urine or serum is the most utilized indicator of pregnancy onset. Serial measurements of β-hCG concentration every 48 h are generally used to confirm early pregnancy, and demonstrate utility in differentiating normal, progressing pregnancies from ectopic pregnancies or spontaneous abortions [3] [4].

The implantation potential of top quality embryos can be derived from human chorionic gonadotropin (hCG) levels in serum [5]. It has been theorized that blastocyst quality is reflective of morphology and that this grade might impact early trophoblastic invasion, which is best observed by evaluating β-hCG serum dynamics [6]. Furthermore, it

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has been demonstrated that embryos with higher trophectoderm (TE) grades secrete β-hCG at an earlier time compared to embryos with poor TE grades. This secretion of hCG by embryos with high TE grades is considered by some researchers to be critical for optimizing implantation and blastocyst-endometrium crosstalk [7] [8].

Multiple studies have assessed differences in morphologic compartments of the human embryo, implantation potential, and/or pregnancy losses after transfer. The evidence derived from these studies remains conflicting. Some studies found that morphologies of the three main compartments (blastocoel expansion, trophectoderm, and inner cell mass) of the blastocyst were positively associated with ART treatment outcomes. For example, studies based on using a fresh blastocyst transfer approach found that ICM was more associated with implantation and clinical pregnancy rates while TE grade had no effect overall in these outcomes [9] [10] [11]. Conversely, a study published by Van den Abbeel et al. showed that expansion grade was the most significant embryonic compartment associated with pregnancy outcomes. Although those same authors concluded that TE grade was most related with treatment outcomes [12], it is important to mention that prior research about this subject based their findings on fresh embryos transfer that included more than one blastocyst selected for transfer. Furthermore, no chromosomal analysis of the embryos was performed. A study by Nazem et al. evaluated single euploid embryo transfers and demonstrated that ICM as the best predictor of sustained implantation. However, the same study concluded a composite score of all compartments of the blastocyst and the day of the embryo biopsy may prove to be additionally beneficial towards selection prior to transfer [13]. Finally, other researchers have found that neither the ICM nor the expansion grade, but the morphological appearance of TE cells, was the most impactful parameter in predicting ART treatment success [14] [15], although those studies did not include genetically screened blastocysts in the study design and those embryo transfers were performed in stimulated endometrium after controlled ovarian stimulation cycles.

Our study aims to analyze whether TE grade can be used as an ancillary marker of embryo quality, and if so, does it correlate with initial β-hCG levels, a known marker of implantation potential. Few reports have shown variability in initial β-hCG values with differences in embryonic or cycle characteristics such as embryo morphology score [6], the day of embryo transfer [16], and/or type of embryo transfers (i.e., fresh vs. frozen) [17] [18]. Unfortunately, no publication has yet to account for different days and assays for hCG measurement, multiple embryos transferred, distinct embryo grading combinations, and/or within a robust sample size. To date, all published data about this topic has yet to include the assessment of initial measurement of β-hCG levels after transfer of euploid blastocysts; a fact in which we deem to be of the utmost importance when analyzing the characteristics of the implantation and early human development.

This study aimed to analyze if there is a correlation between blastocyst TE grading with the initial measurement of serum β-hCG within 9 days of a single euploid embryo transfer. Also, we explored whether TE grade associates with β-hCG duplication 48 h after first measurement. Last, our study aims to explore if there is any correlation between TE grading with subsequent pregnancy outcomes.

Material and methods

Study design and patient populations

The retrospective cohort analysis was performed at a single center and included infertility patients who underwent an IVF cycle with PGT-A with a subsequent single euploid embryo transfer (SEET), from September 2016 to November 2021. All patients underwent controlled ovarian stimulation (COH), intracytoplasmic sperm injection (ICSI), extended embryo culture, TE biopsy, and preimplantation genetic testing for aneuploidy (PGT-A). All PGT-A analyses were performed with next-generation sequencing (NGS) technology; all COH and laboratory methods had been previously described [19]. Only cases with a positive pregnancy test defined as a β-hCG value of ≥ 2.5 mIU/mL in serum measured 9 days after the embryo transfer were included in the analysis. Also, only cases that underwent repeated β-hCG 48 h after the first measurement (11 days after ET) were included. Venous blood samples were collected every time before 1000 h on days 9 and 11 after blastocyst transfer. All β-hCG measurements were performed using electrochemiluminescence immunoassays (ECLIA) (Immulite 2000; Siemens Medical Solutions Diagnostics, California [measuring range 0.4–5,000 mIU/mL. Sensitivity of the assay 0.4 mIU/mL. Intra-assay coefficient of variation 6.6% at concentration of 6.5 mIU/mL and 2.5% at 3,120 mIU/mL] and Cobas e-601; Roche Diagnostics GmbH, Mannheim [measuring ranges 0.2–10,000 mIU/mL. Sensitivity of the assay 0.1 mIU/mL. Intra-assay coefficient of variation 4.2% at concentration of 1.1 mIU/mL and coefficient of variation 2.1% at concentrations of 9,672 mIU/mL]).

Cases having measured β-hCG levels in different days or with incomplete information were excluded from the analysis, also cases involving multiple TE biopsies, multiple thaw/freeze procedures, and cases with patients utilizing donor oocytes, testicular sperm extraction, and/or patients with known chromosomal rearrangements were excluded from analysis. Finally, cases that presented monozygotic splitting, ectopic pregnancies, or cases that were suspected as pregnancies of unknown location were excluded from the analysis.

Blastocysts were graded based on a proprietary, center-specific scoring system (modified Gardner's System) [20] [21] for blastocyst intended to undergo TE biopsy for PGT. Grading was performed only by senior embryologists at the fertility center. The degree of expansion was defined as follows: 1 = early blastocyst: cavity beginning to form, 2 = early blastocyst: cavity is < 50% of the volume of the embryo, 3 = full blastocyst: cavity completely fills the embryo, 4 = expanded blastocyst: cavity volume has exceeded volume of embryo in zona resulting in at least four to five cells herniating out of zona, 5 = hatching blastocyst: 50% or more of TE is herniating through zona, and 6 = hatched blastocyst: blastocyst completely escaped from the zona. The inner cell mass (ICM) grading was determined as follows: A = many cells tightly compacted, B = some cells tightly compacted or organizing, C = some cells disorganized, and D = few cells disorganized. TE was graded as follows: A = many cells forming a cohesive epithelium, B = moderate cells forming a loose epithelium, C = some cells forming a loose epithelium, and D = very few cells. For the purpose of this analysis, all subjects were categorized in three cohorts based on the TE grade at time of embryo transfer (TE A, TE B, and TE C).

For the embryo transfer procedure, all cases underwent synthetic endometrial preparation as previously described [13]. For all clinical cases, thawing and transfer of the embryos was carried out on the sixth day of progesterone supplementation regardless of the day of embryo development at time of cryopreservation. Euploid embryos with the top morphology grade were selected for transfer. In gender selection for family balancing cases, the highest-graded embryo of the preferred genetic sex was transferred. Embryos biopsied on day 5 were preferentially selected over biopsied day 6 embryos when shared among the same patient and with 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, as described previously [13].

Outcome measures

Primary outcome of the study was to analyze if the first β-hCG measurement in mIU/mL, 9 days after the embryo transfer, correlates with TE grade while adjusting for important co-variables. Secondary outcomes included clinical pregnancy rate (CPR): the proportion of patients with ultrasonographically detectable 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 5th week of pregnancy; clinical pregnancy loss rate (CPL): pregnancy loss occurring after the presence of a confirmed gestational sac; and ongoing pregnancy rate (OPR) sustained pregnancy after detected fetal heart beat 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 [22].

Statistical methods

Statistical analysis was performed using SAS version 9.4 (SAS institute Inc. Cary, NC, USA). Demographic, COH, and embryological data was registered for all participants. Descriptive and comparative unadjusted analysis was performed by ANOVA, Kruskal–Wallis, Fisher exact, and χi^2 tests as appropriate. A logistic regression, analysis of covariance (ANCOVA), and an adjusted mixed effect model with a random intercept were used to evaluate β-hCG levels with regard to TE grade. The regression model was fitted with generalized estimating equations (GEE) to account for patients who underwent multiple COH or FET cycles. Adjusted odd ratios (aOR) with 95% confidence intervals (95% CI) were calculated. All variables that showed significance on the unadjusted analysis and/or variables that thought to be clinically relevant were encompassed and adjusted for as covariates in the models. All *p*-values were two sided with a clinical significance level determined at $p < 0.05$. For secondary outcomes, a power analysis calculation required a sample size of 269 patients per group to detect a difference of 10% in CPR to have an 80% power with an alpha of 0.05. Likewise, for detecting a 10% difference in CPL rates, a sample size per group of 221 was calculated, and finally, for a 10% difference in BPL rates, a sample size of 260 FETs per group were required.

Regulatory approval

This retrospective analysis was approved by an academic Institutional Review Board (HS #: STUDY-18–00,441). Patient information was de-identified before data analysis.

Results

A total of 2,798 positive pregnancy cases after a SEET from 2,274 couples that meet the inclusion criteria were assessed within the study period. The overall median β-hCG value 9 days after the SEET was 119.55 mIU/mL inter-quartile range (IQR 56.9, 184.45). The median β-hCG level on day 11 was 301.6 mIU/mL (IQR 130–475.8). A mean percentage increase overall of 152.30% (CI95% 187.46–206.95) was observed after 48 h from first measurement. Of the entire cohort, 76.05% of patients ($n = 2128$) presented an increase of 100% or doubled the levels from the baseline β-hCG value. Among the entire cohort, 81.59% ($n = 2283$)

progressed to a clinical pregnancy, and 67.48% ($n=1888$) progressed to ongoing pregnancy. Of the patients in the study, 18.41% ($n=515$) experienced a biochemical pregnancy loss, and 14.12% ($n=395$) experienced a clinical pregnancy loss.

All patients were categorized in 3 groups based on the TE morphological grade at time of embryo transfer: [TE A ($n=1,077$), TE B ($n=1,165$), and TE C ($n=556$)]. A significant difference in the first β-hCG measurement (day 9 post SEET) among groups was observed (TE A median 143.4 mIU/mL IQR 79.2–211.2; TE B 119 mIU/mL IQR 57.1–177.8; TE C 82.4 mIU/mL IQR 36.3–136.4, $p \leq 0.0001$). Also, significant differences were found in the second β-hCG measurement levels among cohorts (11 days' post transfer) (363.2 mIU/mL IQR 177.9–543.2; 297 mIU/mL IQR 138.4–457; and 212 mIU/mL IQR 84.9–361.5, $p \leq 0.0001$), respectively (Fig. 1). The percentage increase in β-hCG from day 9 to day 11 in the cohorts was comparable (149.7% CI95% 139.3–160; 153.1% CI95 143.0–163.1; 158.1% CI95% 143.3–172.9, $p=0.61$). The proportion of patients in each cohort that doubled β-hCG levels was comparable among each TE group (TE A: 77.3%; TE B: 76.0%; and TE C: 73.5%, $p=0.23$).

When analyzing demographic characteristics of the cohorts, differences were found in the oocyte age in years (35.4 SD 2.8; 36.5 SD 3.9; and 36.0 SD 3.8, $p=0.002$) and anti-Müllerian hormone (AMH) levels (3.7 ng/mL SD 3.8; 3.7 ng/mL SD 4.3; and 3.1 ng/mL SD 3.3), respectively. Other demographic and clinical characteristics were comparable among study groups (Table 1).

During the unadjusted ANCOVA exploration, there was a significant correlation found between the TE grade and the median β-hCG level measurement ($p \leq 0.0001$; $r^2=0.05$). A post hoc comparison with Tukey's HSD test showed constant differences among the multiple group comparisons. Then in a mixed effect model with a random intercept after adjusting for other embryonic morphological compartments (ICM and expansion), day of TE biopsy, oocyte age, patients BMI, AMH, and endometrial thickness at ET and ECLIA equipment, the correlation between the TE grade and first β-hCG levels remained significant ($p \leq 0.0001$, $r^2=0.10$).

IVF outcomes were also compared among the 3 TE groups. There were significant differences in the proportion of cases that progressed to clinical pregnancy among cohorts (TE A: 83.7%, TE B: 76.0%, and TE C: 73.56%, $p=0.01$). Also, ongoing pregnancy rates showed significant differences among groups (70.8%; 66.70%; and 62.5%, $p=0.002$), respectively. While looking at adverse outcomes, biochemical loss rate was found to be significantly different among cohorts being higher on the lower TE grading cohort (TE A: 16.2%; TE B: 18.5%; and TE C: 22.3%, $p=0.01$), but no differences in clinical pregnancy loss rates were found among cohorts (12.91%, 14.76%, and 15.11%, $p=0.34$), respectively (Table 2). In a multivariate regression analysis fitted with a GEE, after adjusting for ICM, expansion grade, day of TE biopsy, oocyte age, BMI, AMH, and endometrial thickness at ET, no association was found between the TE grade and lower odds of achieving clinical pregnancy (TE A vs. TE B: aOR 0.98 CI95% 0.95–1.02; TE A vs. TE C: aOR 0.96 CI95% 0.92–1.01). Also, no association was found between TE grade and odds of achieving ongoing pregnancy (TE A vs. TE B: aOR 0.96 CI95% 0.92–1.01), but there was a significant association with TE C and lower odds of developing to ongoing pregnancy rate (TE A vs. TE C: aOR 0.93 CI95% 0.88–0.99). Furthermore, in the same adjusted analysis, the TE grade was not associated with

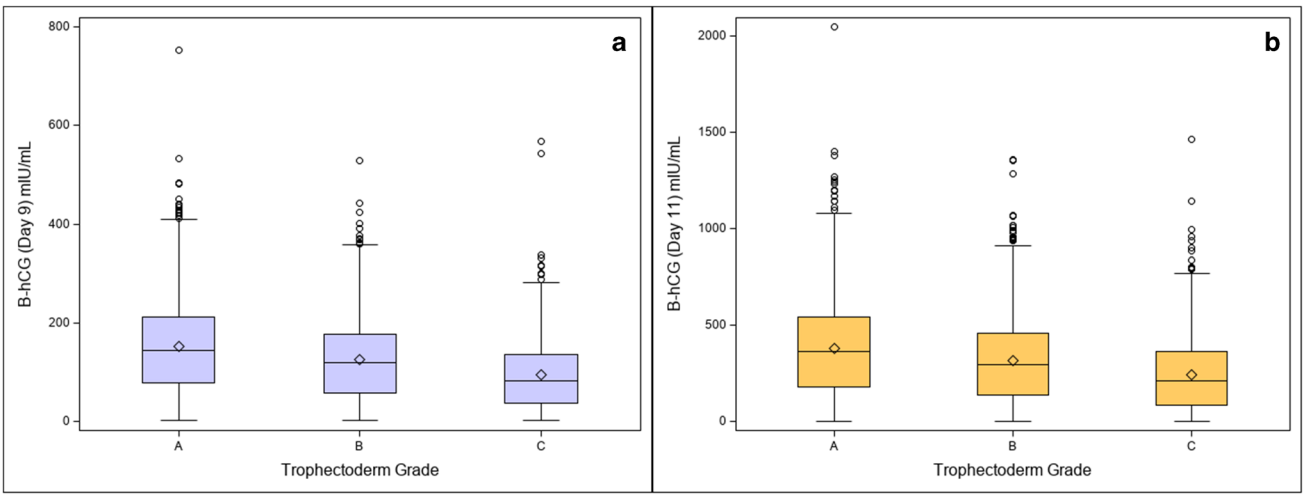

Fig. 1 Box plot showing the differences between different trophectoderm grading groups and their median and interquartile ranges. **a** β-hCG levels per trophectoderm grade measured 9 days after embryo transfer. Kruskal–Wallis test ($p \leq 0.0001$). **b** β-hCG levels per trophectoderm grade measured 11 days after embryo transfer. Kruskal–Wallis test ($p \leq 0.0001$)

Table 1 Demographic characteristics and data comparison between groups based by trophectoderm grading

	Trophectoderm grade A		Trophectoderm grade B		Trophectoderm grade C		Test statistic
	N = 1077		*N* = 1165		*N* = 556		
	Mean/median	SD/IQR	Mean/median	SD/IQR	Mean/median	SD/IQR	
β-hCG on day 9 post ET (mIU/mL)	143.4	79.2–211.2	119	57.1–177.8	82.4	36.3–136.4	< 0.0001
β-hCG on day 11 post ET (mIU/mL)	363.2	177.9–543.2	297	138.4–457	212	84.9–361.5	< 0.0001
Percentage β-hCG increase (%)	129.56	80.85	133.21	87.61	131.44	97.86	0.61
Age (years)	35.96	3.89	36.56	4.01	36.82	3.97	< 0.0001
Oocyte age (years)	35.48	3.86	35.95	3.98	36.08	3.89	0.002
Body mass index	24.02	4.36	23.74	4.35	23.87	4.57	0.34
Anti-Mullerian hormone (ng/mL)	3.77	3.87	3.73	4.31	3.19	3.37	0.03
Baseline progesterone (ng/mL)	0.43	0.20	0.42	0.26	0.43	0.26	0.40
Baseline antral follicle count	11.21	8.32	11.31	8.47	10.68	7.43	0.36
Estradiol days before convertion	13.65	3.02	13.98	3.22	13.75	3.19	0.04
Serum estradiol at conversion (pg/mL)	344.87	386.06	315.65	316.51	326.04	385.36	0.15
Endometrial thickness at ET (mm)	9.66	3.58	9.47	2.11	9.36	2.15	0.08
Serum progesterone after ET (ng/mL)	28.64	10.52	28.13	13.73	28.95	11.07	0.36
Previous vaginal oocyte retrievals	1.52	0.94	1.57	0.95	1.61	0.94	0.17
Gravidity	1.05	1.28	1.17	1.39	1.14	1.24	0.11
Parity	0.41	0.69	0.45	0.70	0.46	0.68	0.22

Data presented as mean and SD, or median and interquartile range (IQR), unless stated otherwise. Statistical significance, $p < 0.05$

Table 2 Unadjusted comparison of clinical IVF outcomes among trophectoderm grade groups

	Trophectoderm grade A		Trophectoderm grade B		Trophectoderm grade C		*p*-value
	N = 1077		*N* = 1165		*N* = 556		
	N	%	*N*	%	*N*	%	
Proportion of cases that had an β-hCG increase > 100% (48 h difference)	833/1077	77.34	886/1165	76.05	409/556	73.56	0.23
Clinical pregnancy rate	902/1077	83.75	949/1165	81.46	432/556	77.70	0.01
Ongoing pregnancy rate	763/1077	70.84	777/1165	66.70	348/556	62.59	0.002
Biochemical pregnancy loss rate	175/1077	16.25	216/1165	18.54	124/556	22.30	0.01
Clinical pregnancy loss rate	139/1077	12.91	172/1165	14.76	84/556	15.11	0.34

Data presented as sample size (*N*) and proportions (%), unless stated otherwise. Statistical significance, $p < 0.05$

higher odds of biochemical pregnancy loss (TE A vs. TE B: aOR 1.01 CI95% 0.97–1.05; TE A vs. TE C: aOR 1.03 CI95% 0.98–1.08), and no association was found between TE grade and higher odds of clinical pregnancy loss (TE A vs. TE B: aOR 1.02 CI95% 0.98–1.05; TE A vs. TE C: aOR 1.03 CI95% 0.98–1.07).

An analysis of β-hCG dynamics from the first (day 9 after ET) to the second measurement (day 11 after ET) was also performed. We classified the groups based on their magnitude changes into 3 groups: group 1: if β-hCG declined on day 11; group 2: β-hCG increased < 100% after 48 h from first measurement; group 3: β-hCG > 100% in 48 h from first measurement (Table 3). Statistical differences in pregnancy outcomes were found among the pregnancy outcomes in the 3 cohorts (Table 3). In patients that experienced β-hCG decline, the biochemical pregnancy loss rate was 99.7%, clinical pregnancy loss rate was 0%, proportion of pregnancies that progressed to clinical pregnancy were 0.3%, and the ongoing pregnancy rate achieved was 0.3% (only 1 case developed into ongoing pregnancy). In patients where β-hCG levels increased < 100%, biochemical pregnancy loss rate was 34.9%, clinical pregnancy loss rate was 23.5%, clinical pregnancy achieved was 65.1%, and ongoing pregnancy rate of 41.6% was found. In patients where β-hCG levels

Table 3 Analysis by β-hCG dynamics from the first (day 9 after ET) to the second measurement (day 11 after ET), the groups were classified based on their magnitude changes: group 1: if β-hCG declined or was measured below the first measurement on day 11; group 2: β-hCG presented a "plateau" or had an increase < 100% in 48 h difference, and group 3: if hCG value doubled at least 100% in 48 h difference

HCG dynamic group	Clinical pregnancy		Ongoing pregnancy		Biochemical pregnancy loss		Clinical pregnancy loss	
	N	%	*N*	%	*N*	%	*N*	%
Declined	1/326	0.3%	1/326	0.3%	325/326	99.7%	0/326	0.0%
Plateaued	224/344	65.1%	143/344	41.6%	120/344	34.9%	81/344	23.5%
Doubled	2058/2128	96.7%	1744/2128	82.0%	70/2128	3.3%	314/2128	14.8%
Difference χi^2	< 0.0001		< 0.0001		< 0.0001		< 0.0001	

Data presented as sample size (*N*) and proportions (%), unless stated otherwise. Statistical significance, $p < 0.05$

more than doubled, the biochemical pregnancy loss rate was 3.3%, clinical pregnancy loss rate was 14.8%, pregnancies that progressed to clinical pregnancy were 96.7%, and an ongoing pregnancy rate of 82.0% was found (Table 3).

Furthermore, a subanalysis looking at the day of TE biopsy and β-hCG rise from day 9 to day 11 found no differences in the mean percentage of serum β-hCG increase among groups: day 5 biopsy (mean β-hCG increase 129.0 SD 82.2), day 6 biopsy (mean β-hCG increase 135.3 SD 93.7), and day 7 biopsy (mean β-hCG increase 133.1 SD 108.7) ($p = 0.18$).

A last subanalysis looking at 1622 negative pregnancy tests after a SEET found that lower TE grade was significantly associated with increased frequency of failed implantation rates: TE A: 32.1%, TE B: 35.63%, and TE C: 46.4% ($p \leq 0.0001$). A multivariate analysis that used the best trophectoderm grading (TE A) as a reference and after adjusting for oocyte age, BMI, AMH, endometrial thickness, and embryonic ICM, expansion grades and day of embryonic biopsy showed a significant association with lower TE grading and increased odds of failing implantation: TE A vs. TE B: aOR 1.15 CI95% 0.9–1.3; and TE A vs. TE C: aOR 1.60 CI95% 1.34–1.91.

Discussion

In vitro fertilization with a single euploid embryo transfer continues to be an exceptional model to investigate the role of early serum β-hCG levels on pregnancy outcomes, especially since it can allow clinicians to properly date embryonic age [23]. Our study findings suggest that trophectoderm morphological grading is correlated with serum β-hCG levels measured 9 days after a single euploid embryo transfer; even after adjusting for other embryonic compartments and/or patient clinical features. We propose that this data helps validating the link between morphology assessment and early embryo development in vivo.

Few studies had investigated the relationship between trophectoderm morphology grade and the earliest levels of detectable serum β-hCG. Some studies had found that the expression of hCG is closely related to trophoblast differentiation [24] [25] potentially elucidating why optimal trophectoderm grade, which encompasses higher count and better organization of cells, might associate with a better ability to produce hCG [6] [16] [26]. This could translate into a strengthened signaling capacity for blastocyst implantation potential [27]. Also, other studies have shown embryos with higher TE grades secrete hCG earlier than poor TE grade counterparts, which might give these embryos an advantage in achieving implantation and optimizing endometrial crosstalk [18].

Although the valuable insights obtained from those landmark studies have accelerated our knowledge regarding early hCG dynamics, most were based on findings of unscreened blastocysts, cleavage stage embryos, multiple embryo transfers, or did not separate cohorts based on embryonic quality nor embryo compartments. Our study differentiates from those studies by evaluating the impact of the TE grade and early β-hCG levels by adjusting for the other remaining blastocyst compartments. Also and more importantly, our study is the first one that analyzed only blastocyst-stage embryos identified to be euploid via NGS technology and included only single euploid embryo transfers over synthetically prepared endometrial linings. Furthermore, we performed a statistically adjusted model in an attempt to understand the pure effect of TE grading on β-hCG dynamics. Our findings suggested a clinically discrete but significant association between optimal TE grading embryos and higher β-hCG levels in the first serum measurements. It is important to mention that our study findings might not be generalizable for natural conceptions [28] or non-biopsied blastocyst transfers. Some other studies proposed that β-hCG levels can be impacted by other factors such as the utilization of frozen or fresh embryos [29] or that trophectoderm biopsy removes a small portion of cells of the entire blastocyst but has been

demonstrated to yield lower mean hCG values compared with non-biopsied blastocysts. In this last case, some authors had suggested that the threshold cutoff value for predicting successful pregnancies in these PGT cases should be set at lower levels compared with non-biopsied cases [30].

Majority of clinical studies looking at early β-hCG dynamics during ART treatment have focused on the predicting value of these serum measurements to foresee different pregnancy outcomes. Our findings suggest that — in the general infertile population — after adjusting for important co-variables, there is a significant association of lower quality TE grades and decreased odds of achieving embryo implantation and ongoing pregnancy being consistent with previously published reports [15] [31] [32]. Nevertheless, when looking at adverse outcomes such as biochemical pregnancy loss or clinical pregnancy loss, after adjusting for other compartments of embryo grade (expansion and ICM) and patient characteristics, our study failed to detect an association between inferior TE grade and increased odds of biochemical and clinical pregnancy loss rates. This finding corroborates with other published analyses, which can be reassuring information to patients [10] [11]. We highlight a single compartment of the blastocyst morphology is not at the helm of the whole process of implantation. As our group previously demonstrated, every compartment of the human embryo has impact on the odds of implantation and pregnancy outcomes. Yet, a composite score including all compartments and day of TE biopsy could yield additional guidance in embryo selection prior to transfer [13].

Finally, in a subanalysis we analyzed IVF outcomes based on hCG dynamics in the first 2 measurements obtained on 9 and 11 days after ET, we observed significant differences among the pregnancy outcomes based on the categorization of patients that declined, plateaued, or doubled up their β-hCG levels. Our study analysis suggested that patients who did not present a 100% increase of the initial value after 48 h or patients who had a decrease on the baseline hCG levels had critically significant higher odds of presenting a biochemical or clinical pregnancy loss. Similar findings had been previously reported on different embryo transfer settings such as day 3 cleavage stage or single blastocyst transfers [3] [4] [5] [33] [34].

Our study is not without limitations. The retrospective nature the analysis comes along with increased chances of selection bias, although the calculated sample sizes and power analyses showed that the sample size of our populations could suffice to detect significant differences in pregnancy outcomes among cohorts. Also besides potential demographic differences in the groups analyzed by TE grading, our study performed an adjusted analysis controlling for important patient and embryonic characteristics in order to isolate the effects of TE characteristics and initial β-hCG levels and furthermore pregnancy outcomes.

Another potential limitation in the generalizability of our findings is the cutoff value for determining a positive pregnancy. The study center establishes a positive pregnancy at a serum level of β-hCG of ≥ 2.5 mIU/mL. It has been described previously that this cutoff value could discriminate between pregnancy and non-pregnancy in non-menopausal patients with a sensitivity of 90.8% and a specificity of 96.7% [35] [36] and represents the 97.5% percentile of hCG concentration ranges for premenopausal non-pregnant patients [37]. Our cutoff might differ from other laboratories. We note that unlike previous publications which utilized different hCG measuring assays, our study assays are consistent and highly sensitive which has been tested to offer the best detection rates of the different hCG molecules, including hyper glycosylated hCG, the principal hCG molecule in early pregnancy [38]. Also, it is important to note that our results should be interpreted with caution because other reference centers or laboratories might utilize different assays as the ones utilized in this study, a factor that can limit the generalizability of our findings due to inter-assay discordances [39].

Furthermore, another important fact to consider is the possibility of false positive hCG determinations due to other hCG producing entities or conditions that could not possibly be excluded in our study design [40]. However, in our population of non-menopausal and relatively young patients, these possibilities appear to be exceptionally rare [41]. Additionally, our study does not describe the perinatal outcomes and live birth outcomes of the entire cohorts, mostly due to the fact that these patients are still pregnant. Additionally, the main objective of this study design was not to analyze perinatal outcomes. Albeit, our group previously has described that embryo TE quality does not correlate with major adverse perinatal outcomes or placental weight at delivery [42]. Finally, although all senior embryologists are trained for morphologic grading in the same fashion, there remains inherent subjectivity in morphologic grading which cannot be accounted for in this study. Based on these limitations, it is difficult to accurately quantify the association between TE and subsequent outcomes on an in vivo setting, although our study proposes an adjusted method that accounts for important factors that were not taken into account in prior studies that aimed to analyze the association between TE grade and IVF outcomes.

In conclusion, after adjusting for clinical parameters, embryonic expansion, and inner cell mass grade, our data showed euploid embryo TE grade correlates with β-hCG levels at first pregnancy test measurement. The ultrastructural appearance of the TE cells in euploid embryos might represent a surrogate marker of embryo's capacity to properly adhere and invade the endometrium during the early implantation process. Furthermore, patients who transferred embryos with poor TE grade were found to experience lower

odds of achieving ongoing pregnancy. Yet, no other significant associations between TE grade with any other IVF outcome were observed. Finally, IVF outcomes including biochemical and pregnancy loss rates differ significantly based on the initial hCG measurement dynamics favoring patients that present a 100% increase in serum levels after 48 h of the first measurement. Further studies focusing on synciotrophoblast and endometrial cellular and molecular interactions or genetic pathways could help reproductive specialists to better understand the mechanisms related to early placentation physiology. Similarly, future studies looking at different human blastoid models and their transcriptomes could help to understand the high complexity and scarcely understood process of human implantation and early embryogenesis.

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Data availability Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

References

1. Liu HC, Jones GS, Jones HW Jr, Rozenwaks Z. Mechanisms and factors of early pregnancy wastage in in-vitro fertilization embryo transfer patients. Fertil Steril. 1988;50:95–101.
2. Sung N, Kwak-Kim J, Koo HS, Yang KM. Serum hCG-b levels of postovulatory day 12 and 14 with the sequential application of hCG-b fold change significantly increased predictability of pregnancy outcome after IVF-ET cycle. J Assist Reprod Genet. 2016;33:1185–94.
3. Lambers MJ, van Weering HGI, van't Grunewold MS, et al. Optimizing hCG cutoff values: a single determination on day 14 or 15 is sufficient for a reliable prediction of pregnancy outcome. Eur J Obstet Gynecol Reprod Biol. 2006;127:94–8.
4. Chung K, Sammel MD, Coutifaris C, et al. Defining the rise of serum HCG in viable pregnancies achieved through use of IVF. Hum Reprod. 2006;21:823–8.
5. De Neubourg D, Gerris J, Mangelschots K, Van Royen E, Vercruyssen M, Elseviers M. Single top quality embryo transfer as a model for prediction of early pregnancy outcome. Hum Reprod. 2004;19(6):1476–9.
6. Girard JM, Simorre M, Leperlier F, Reignier A, Lefebvre T, Barrière P, Fréour T. Association between early βhCG kinetics, blastocyst morphology and pregnancy outcome in a single-blastocyst transfer program. Eur J Obstet Gynecol Reprod Biol. 2018;225:189–93. https://doi.org/10.1016/j.ejogrb.2018.04.037.
7. Licht P, Russu V, Lehmeyer S, Wildt L. Molecular aspects of direct LH/hCG effects on human endometrium—lessons from intrauterine microdialysis in the human female in vivo. Reprod Biol. 2001;1:10–9.
8. Tsampalas M, Gridelet V, Berndt S, Foidart JM, Geenen V, Perrier DS. Human chorionic gonadotropin: a hormone with immunological and angiogenic properties. J Reprod Immunol. 2010;85:93–8.
9. Richter KS, Harris DC, Daneshmand ST, Shapiro BS. Quantitative grading of a human blastocyst: optimal inner cell mass size and shape. Fertil Steril. 2001;76:1157–67.
10. Kovacic B, Vlaisavljevic V, Reljic M, Cizek-Sajko M. Developmental capacity of different morphological types of day 5 human morulae and blastocysts. Reprod Biomed Online. 2004;8:687–94.
11. Shapiro BS, Daneshmand ST, Garner FC, Aguirre M, Thomas S. Large blastocyst diameter, early blastulation, and low preovulatory serum progesterone are dominant predictors of clinical pregnancy in fresh autologous cycles. Fertil Steril. 2008;90:302–9.
12. Van den Abbeel E, Balaban B, Ziebe S, et al. Association between blastocyst morphology and outcome of single-blastocyst transfer. Reprod Biomed Online. 2013;27:353–61.
13. Nazem TG, Sekhon L, Lee JA, Overbey J, Pan S, Duke M, Briton-Jones C, Whitehouse M, Copperman AB, Stein DE. The correlation between morphology and implantation of euploid human blastocysts. Reprod Biomed Online. 2019;38(2):169–76. https://doi.org/10.1016/j.rbmo.2018.10.007.
14. Ahlström A, Westin C, Reismer E, Wikland M, Hardarson T. Trophectoderm morphology: an important parameter for predicting live birth after single blastocyst transfer. Hum Reprod. 2011;26:3289–96.
15. Zaninovic N, Berrios R, Clarke RN, Bodine R, Ye Z, Veeck LL. Blastocyst expansion, inner cell mass (ICM) formation, and trophectoderm (TM) quality: is one more important for implantation? Fertil Steril. 2001;76:S8.
16. Dokras A, Sargent IL, Barlow DH. Human blastocyst grading: an indicator of developmental potential? Hum Reprod. 1993;8:2119–27.
17. Oron G, Shavit T, Esh-Broder E, Weon-Young S, Tulandi T, Holzer H. Predictive value of serum HCG concentrations in pregnancies achieved after single fresh or vitrified-warmed blastocyst transfer. Reprod Biomed Online. 2017;35:272–8.
18 Licht P, Russu V, Wildt L. On the role of human chorionic gonadotropin (hCG) in the embryo-endometrial microenvironment: implications for differentiation and implantation. Semin Reprod Med. 2001;19:37–47.
19. Hernandez-Nieto C, Lee JA, Alkon-Meadows T, Luna-Rojas M, Mukherjee T, Copperman AB, Sandler B. Late follicular phase progesterone elevation during ovarian stimulation is not associated with decreased implantation of chromosomally screened embryos in thaw cycles. Hum Reprod. 2020;35(8):1889–99. https://doi.org/10.1093/humrep/deaa123.
20. Veeck LL, Bodine R, Clarke RN, Berrios R, Libraro J, Moschini RM, Zaninovic N, Rosenwaks Z. High pregnancy rates can be achieved after freezing and thawing human blastocysts. Fertil Steril. 2004;82(5):1418–27. https://doi.org/10.1016/j.fertnstert.2004.03.068.
21. Hernandez-Nieto C, Lee JA, Slifkin R, Sandler B, Copperman AB, Flisser E. What is the reproductive potential of day 7 euploid embryos? Hum Reprod. 2019;34(9):1697–706. https://doi.org/10.1093/humrep/dez129.
22. Zegers-Hochschild F, Adamson GD, Dyer S, Racowsky C, de Mouzon J, Sokol R, Rienzi L, Sunde A, Schmidt L, Cooke ID, Simpson JL, van der Poel S. The International Glossary on Infertility and Fertility Care, 2017. Hum Reprod. 2017;32(9):1786–801. https://doi.org/10.1093/humrep/dex234.
23. Al Mamari N, Al Zawawi N, Khayat S, Badeghiesh A, Son WY, Dahan MH. Revisiting serum β-hCG cut-off levels and pregnancy outcomes using single embryo transfer. J Assist Reprod Genet. 2019;36(11):2307–13. https://doi.org/10.1007/s10815-019-01583-x (Epub 2019 Oct 12).
24. Hoshina M, Boothby M, Hussa R, Patillo R, Camel HM, Boime I. Linkage of human chorionic gonadotrophin and placental lactogen biosynthesis to trophoblast differentiation and tumorigenesis. Placenta. 1985;6:163–72.
25. Shi QJ, Lei ZM, Rao CV, Lin J. Novel role of human chorionic gonadotropin in differentiation of human cytotrophoblasts. Endocrinology. 1993;132:1387–95.

26. Butler SA, Luttoo J, Freire MO, Abban TK, Borrelli PT, Iles RK. Human chorionic gonadotropin (hCG) in the secretome of cultured embryos: hyperglycosylated hCG and hCG-free beta subunit are potential markers for infertility management and treatment. Reprod Sci. 2013;20(9):1038–45. https://doi.org/10.1177/1933719112472739 (Epub 2013 Feb 25).
27. Hill MJ, Richter KS, Heitmann RJ, Graham JR, Tucker MJ, DeCherney AH, Browne PE, Levens ED. Trophectoderm grade predicts outcomes of single-blastocyst transfers. Fertil Steril. 2013;99(5):1283-1289.e1. https://doi.org/10.1016/j.fertnstert.2012.12.003.
28. Dokras A, Sargent IL, Gardner RL, Barlow DH. Human trophectoderm biopsy and secretion of chorionic gonadotrophin. Hum Reprod. 1991;6(10):1453–9. https://doi.org/10.1093/oxfordjournals.humrep.a137288.
29. Sites CK, St Marie P, Rahil T. Implantation of fresh and thawed-warmed embryos in single embryo transfer cycles: interpreting the initial beta-HCG. Reprod Biomed Online. 2015;30(3):319–21. https://doi.org/10.1016/j.rbmo.2014.12.003.
30. Lu MM, Wen YX, Liu YL, Ding CH, Zhou CQ, Xu YW. Trophectoderm biopsy reduces the level of serum β-human chorionic gonadotropin in early pregnancy. Fertil Steril. 2020;114(4):801–8. https://doi.org/10.1016/j.fertnstert.2020.05.015 (Epub 2020 Jul 31).
31. Ahlström A, Westin C, Reismer E, Wikland M, Hardarson T. Trophectoderm morphology: an important parameter for predicting live birth after single blastocyst transfer. Hum Reprod. 2011;26(12):3289–96. https://doi.org/10.1093/humrep/der325.
32. Van den Abbeel E, Balaban B, Ziebe S, Lundin K, Cuesta MJ, Klein BM, Helmgaard L, Arce JC. Association between blastocyst morphology and outcome of single-blastocyst transfer. Reprod Biomed Online. 2013;27(4):353–61. https://doi.org/10.1016/j.rbmo.2013.07.006.
33. Poikkeus P, Hiilesmaa V, Tiitinen A. Serum HCG 12 days after embryo transfer in predicting pregnancy outcome. Hum Reprod. 2002;17(7):1901–5. https://doi.org/10.1093/humrep/17.7.1901.
34. Ochsenkühn R, Arzberger A, von Schönfeldt V, Engel J, Thaler CJ, Noss U. Predictive value of early serum beta-hCG levels after single blastocyst transfer. Acta Obstet Gynecol Scand. 2009;88(12):1382–8. https://doi.org/10.3109/00016340903322743.
35. Stenman UH, Alfthan H, Ranta T, Vartiainen E, Jalkanen J, Seppälä M. Serum levels of human chorionic gonadotropin in nonpregnant women and men are modulated by gonadotropin-releasing hormone and sex steroids. J Clin Endocrinol Metab. 1987;64(4):730–6. https://doi.org/10.1210/jcem-64-4-730.
36. Wang Q, Zhang R, Jia M, Luo L, Ding C, Li TC, Zhou C. Serum human chorionic gonadotropin measured 7 days following day 3 embryo transfer might predict pregnancy outcome in IVF. Gynecol Endocrinol. 2017;33(1):62–6. https://doi.org/10.1080/09513590.2016.1203894.
37. Snyder JA, Haymond S, Parvin CA, Gronowski AM, Grenache DG. Diagnostic considerations in the measurement of human chorionic gonadotropin in aging women. Clin Chem. 2005;51(10):1830–5. https://doi.org/10.1373/clinchem.2005.053595 (Epub 2005 Aug 11).
38. Cole LA, DuToit S, Higgins TN. Total hCG tests. Clin Chim Acta. 2011;412(23–24):2216–22. https://doi.org/10.1016/j.cca.2011.08.006.
39. Cole LA. Immunoassay of human chorionic gonadotropin, its free subunits, and metabolites. Clin Chem. 1997;43(12):2233–43 (PMID: 9439438).
40. Braunstein GD. False-positive serum human chorionic gonadotropin results: causes, characteristics, and recognition. Am J Obstet Gynecol. 2002;187(1):217–24. https://doi.org/10.1067/mob.2002.124284.
41. Montagnana M, Trenti T, Aloe R, Cervellin G, Lippi G. Human chorionic gonadotropin in pregnancy diagnostics. Clin Chim Acta. 2011;412(17–18):1515–20. https://doi.org/10.1016/j.cca.2011.05.025.
42. Herlihy N, Sekhon L, Nazem T, Hernandez-Nieto C, Oliva M, Lee J, Sandler B, Mukherjee T, Copperman A. Does morphologic grading of embryonic trophectoderm correlate with quality of placentation and perinatal outcome. Fertil Steril, (2017) 108.