Vietnam Journal of Biotechnology **23**(3): 275-295, 2025. DOI: 10.15625/vjbt-22764

PREDICTION OF PREGNANCY OUTCOMES OF SINGLE VITRIFIED-WARMED BLASTOCYST TRANSFER USING A COMBINATION OF THE EARLY EMBRYO VIABILITY ASSESSMENT AND MORPHOLOGICAL ASSESSMENT

Dinh Hop Vu[1,2,✉], Thi Tu Anh Phi[1], Manh Cuong An[1], Thi Minh Thu Nguyen[1], Thi Lien Huong Nguyen[1], Hoang Le[1] and Van Hanh Nguyen[3,4,✉]

[1]*Assisted Reproductive Technology Center, Tam Anh General Hospital, 108 Hoang Nhu Tiep, Bo De, Hanoi, Vietnam*

[2]*Graduate University of Science and Technology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Nghia Do, Hanoi, Vietnam*

[3]*Institute of Biology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Nghia Do, Hanoi, Vietnam*

[4]*VNU University of Engineering and Technology, 144 Xuan Thuy, Dich Vong Hau, Hanoi, Vietnam*

✉To whom correspondence should be addressed. Email: hopvd@tamanhhospital.vn and nvhanh@vnu.edu.vn

Received: 27.04.2025
Accepted: 21.08.2025

ABSTRACT

Morphological assessment is still the gold standard for embryo selection. Artificial Intelligence (AI) has been developed for embryo selection. However, AI is still a complementary method of supporting humans. This study aims to investigate a combination of the Early Embryo Viability Assessment (EEVA) AI and blastocyst morphological assessment as a predictor of pregnancy outcomes of single vitrified-warmed blastocyst transfer. The retrospective cohort study was conducted in a single center from 2020 to 2023 and included 511 single vitrified-warmed blastocyst transfer cycles. Blastocyst transfer quality was based on morphology. Embryos on Day 3 were evaluated using the EEVA system. The correlation between the EEVA system alone, blastocyst morphological assessment alone or a combination of the EEVA system and blastocyst morphological assessment, and pregnancy outcomes was qualified by GEEs. Comparison of 3 methods to evaluate the results of predicting pregnancy outcomes using the area under the curve (AUC): performed on the prediction probability scale of the model rule. The GEE model using the EEVA system showed a negative association between higher EEVA scores and the likelihood of achieving pregnancy outcomes. Embryos with the highest EEVA score (EEVA 5) have substantially lower odds of achieving successful implantation and ongoing pregnancy compared with those with the lowest score (EEVA 1). The OR of Score 5 vs 1 was 0.282 (95% CI 0.125–0.636, $p < 0.001$) for implantation and 0.228 (95% CI 0.092-0.563, $p < 0.001$) for ongoing pregnancy. The AUC of the GEE model using the EEVA system for

implantation and ongoing pregnancy potential was 0.651 and 0.655, respectively. The AUC of the GEE model using the blastocyst morphological assessment for implantation and ongoing pregnancy potential was 0.703 and 0.700, respectively. The AUC of the GEE model combining both systems for implantation and ongoing pregnancy potential was 0.730 and 0.726. The differences were statistically significant ($p < 0.001$). The EEVA system can predict the success rates, especially when combining EEVA with blastocyst morphological assessment in blastocyst selection for transfer.

Keywords: Automated embryo assessment, blastocyst, Early Embryo Viability Assessment (EEVA), morphology, time-lapse Genea Embryo Review Incubator (GERI).

INTRODUCTION

Currently, single blastocyst transfer is a trend at the *in vitro* fertilization (IVF) center to avoid multiple pregnancies. Therefore, single vitrified-warmed blastocyst transfer (SVBT) is the main strategy in the embryo transfer policy of many centers (Kato *et al*., 2018). The selection of embryos for transfer based on morphology is often subjective among embryologists, between different embryo morphology assessment systems, and between different times of assessment (Aparicio *et al*., 2013). As a result, these techniques are not very precise, resulting in variable and sometimes inaccurate embryo ranking and selection for transfer (Gallego *et al*., 2019).

To improve embryo culture and selection, the time-lapse incubator system was introduced to allow continuous monitoring of embryos, which provides a lot of information about the embryo's development process for the assessment of embryologists without removing embryos from the stable culture environment of the incubator (Aparicio *et al*., 2013; Kaser & Racowsky, 2014; Sundvall *et al*., 2013; Wong *et al*., 2013).

Although the time-lapse system has greatly improved embryo evaluation, it requires highly qualified embryologists and can be complex and time-consuming. Furthermore, although subjectivity is reduced, evaluation still requires manual annotation, especially when many new parameters are used (Bori *et al*., 2020). Artificial intelligence (AI) can overcome these issues in selecting embryos for transfer.

Recently, the benefits of AI in predicting pregnancy outcomes have been widely studied (Bori *et al*., 2021; Bormann *et al*., 2020; Swain *et al*., 2020; Zaninovic & Rosenwaks, 2020). The first automatic system for the analysis of images obtained through time-lapse was EEVA (Early Embryo Viability Assessment) (VerMilyea *et al*., 2014). This technology was the first practical application of artificial intelligence in an IVF laboratory, and it solves many of the previously described problems with the introduction of automation. EEVA is an automated embryo assessment software at the early embryo development stage, integrated exclusively into the time-lapse Genea Embryo Review Incubator (GERI). This is the first software approved by the US Food and Drug Administration (Diamond *et al*., 2015). The EEVA system records the development of each embryo with a cell-tracking system and predicts the likelihood (high, medium, or low) that an embryo will form a blastocyst based on automated detection and analysis of time-lapse imaging information during the early cell division stage. The EEVA system automatically

detects the durations of the 2-cell (P2; t3–t2) and 3-cell (P3; t4–t3) stages and characteristics of the embryo during the first 3 days of embryonic development. This may avoid prolonging culture and save resources (Revelli *et al*., 2019). EEVA assesses embryos automatically and classifies them into 5 degrees: E1, E2, E3, E4, and E5, with a decreasing ability to develop into the blastocyst stage (Diamond *et al*., 2015).

Although the EEVA system was originally developed to predict embryos' potential to reach the blastocyst stage at early developmental stages, strong associations were also found between the best EEVA categories and increased implantation rates in transferred embryos (Aparicio-Ruiz *et al*., 2016; VerMilyea *et al*., 2014). However, some studies showed no difference in the selection of embryos for implantation when using the EEVA system alone or when EEVA was combined with morphological assessment (Kaser & Racowsky, 2014; Kieslinger *et al*., 2016; Yang *et al*., 2018). Some algorithms developed to predict implantation potential focus on late predictive parameters such as blastocyst morphology and/or timing of blastulation (Desai *et al*., 2016; Goodman *et al*., 2016; Mizobe *et al*., 2017) and KIDScore D5 (Vitrolife, Denmark); some algorithms also include early morphokinetic parameters (Gallego *et al*., 2019). On the other hand, the relationship between pregnancy outcomes and EEVA classification after a single vitrified-warmed blastocyst transfer has not been evaluated. Therefore, the clinical application of EEVA still requires studies that analyze its correlation with pregnancy outcomes.

This study aimed to investigate the EEVA system as a predictor of pregnancy outcomes, such as implantation and ongoing pregnancy. Furthermore, the aim was to compare the performance of the EEVA system with the traditional morphological classification and with the combination of both systems for implantation and ongoing pregnancy potential.

MATERIALS AND METHODS

Study design

This study was designed as a retrospective cohort study, with 511 single vitrified-warmed blastocyst transfer cycles from 2020 to 2023 in a single Assisted Reproductive Center. All embryos were assessed by the EEVA system on Day 3, resulting in classification into scores from E1 (best) to E5 (worst) to predict the ability of blastocyst formation based on the embryo's morphokinetic characteristics. Blastocysts were assessed morphologically before vitrification. Blastocysts were warmed before transfer for at least 1 h. Blastocyst transfer quality had not been reduced in this study. There were no uterine abnormalities in the patient. The embryologist evaluating blastocyst quality was blinded to the EEVA score.

The correlation between univariate variables, including maternal age, endometrial thickness, body mass index (BMI), embryonic age, embryonic expansion degree, inner cell mass (ICM) morphology, trophectoderm (TE) morphology, EEVA system, and pregnancy outcomes, was assessed by generalized estimating equations (GEEs). The GEE model using blastocyst morphological assessment alone, the EEVA system only, and a combination of both systems for predicting implantation

and ongoing pregnancy were compared to each other.

Inclusion criteria

The study included all patients who were indicated for blastocyst culturing in the GERI incubator and transferred a single blastocyst in a vitrified-warmed cycle. After thawing, the blastocyst transfer's quality did not decrease. The embryo transfer patient had an endometrial thickness of 7 mm to 13 mm. There were no uterine abnormalities in the patient, and there was enough information for the research.

Exclusion criteria

Exclusion criteria included oocyte donation, surrogacy, severely abnormal sperm, and preimplantation genetic testing (PGT).

Oocyte and sperm handling

Oocytes after retrieval were collected, washed, and incubated in pre-equilibrated G-IVF PLUS culture medium (Vitrolife, Sweden) at a benchtop BT37 incubator at 37°C using 6% CO_2 and 5% O_2 mixing gas. After incubation (approximately 3 h), oocyte denudation was carried out by mechanical pipetting in G-IVF PLUS medium containing the enzyme hyaluronidase 80 IU/mL. After 2 h of culture in IVF PLUS medium, intracytoplasmic sperm injection (ICSI) was performed in gamete medium (Cook Medical, USA) supplemented with HEPES at 200 × magnification using a Nikon inverted microscope with Hoffman optics.

Sperm used for ICSI were filtered and washed by a concentration gradient method. All oocytes after ICSI were cultured in the continuous single culture NXC plate (Irvine Scientific, USA).

Embryo culture and imaging

Embryos were cultured in the GERI Dishes® with a capacity for up to 16 embryos sharing a common medium droplet of 80 µL of pre-equilibrated continuous single culture-NXC plate (Irvine Scientific, USA) covered in 3.5 mL of mineral oil in the GERI incubator (Genea Biomed, Australia), USA. Embryos were kept inside the incubator uninterruptedly from Day 1 to 3. The fertilization, Day 2, and D3 scores were performed on the GERI incubator's screen. The embryos were assisted in hatching on Day 3, and the culture medium was changed outside the incubator. After assisted hatching, the embryos were cultured uninterrupted until Day 5 and 6.

The EEVA system 3.0 (Progyny Inc., CA, USA) was used to image embryonic development and predict blastocyst formation. The system consists of a microscope (EEVA scope), which was placed in a GERI incubator, and a cell-tracking system for registering cell divisions. Every 5 minutes, a single, high-resolution, single-plane dark field illumination image was taken of each embryo.

Embryo assessment

Evaluation and grading of embryo morphology were according to the Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting, 2011 (Balaban *et al*., 2011). Fertilization was assessed at 17 ± 1 h post-ICSI by the presence of two pronuclei and two polar bodies. The number of blastomeres on embryo Day 3 was assessed at 68 ± 1 h post-ICSI. Blastocyst morphology was manually evaluated by embryologists on Days 5 and 6, based on cavity expansion, quality of TE, and ICM (according to the scoring

blastocysts by the Gardner and Schoolcraft grading system), at 116 ± 1 h (Gardner & Schoolcraft, 1999), (with a hatched blastocyst will be BL 7 for blastocyst expansion).

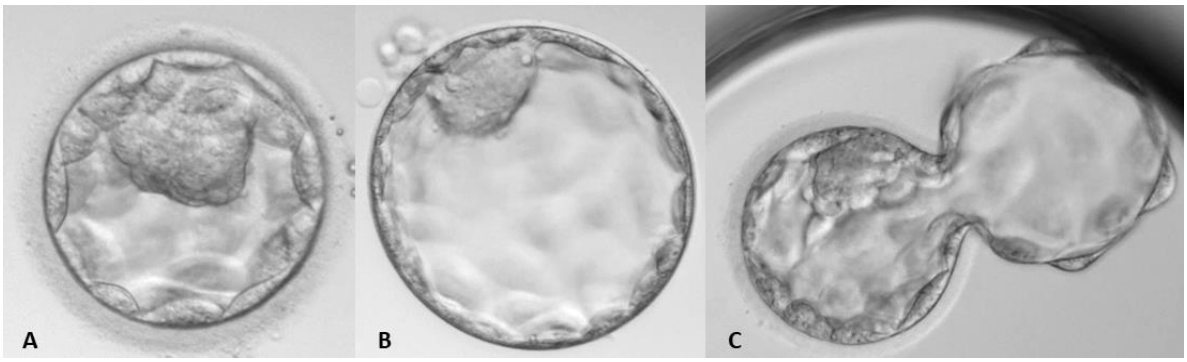


Figure 1. Time-lapse image of blastocyst on Day 5. (A) BL3 AA; (B) BL4 AA; (C) BL5 AA. Scale bar = 20 µm.

The EEVA assessment was based on Day 3 embryo morphokinetics. The EEVA system automatically recognized the durations of the 2-cell (P2; t3–t2) and 3-cell (P3; t4–t3) stages and characteristics of the embryo during the first 3 days of embryonic development. In addition, the number of blastomeres in the Day 3 embryo of each embryo was put into the EEVA system, which then produced the EEVA grade automatically for each embryo. All embryos were classified by the EEVA system (with Version 3.0) and classified with a numeric score from E1 (best) to E5 (worst) according to their likelihood of reaching the blastocyst stage. The EEVA score is calculated based on the age of the oocyte at the retrieval day; some morphokinetics, including the duration of the 2 cells (P2: t3–t2), the duration of the 3 cells (P3: t4–t3); the cell number of embryo Day 3 at 68 h; and some characteristics observed up to 68 h post insemination, which are proprietary to the manufacturer.

Blastocyst vitrification and warming

For vitrification on Day 5 or 6, blastocysts were required to attain a blastocyst expansion degree of 3 (>BL2). These blastocysts were vitrified immediately according to the Cryotop vitrification method (Vitrolife, Cryotop Sweden). If the developing embryo did not fulfill the criteria, it was cultured for a maximum of 7 days.

When the patient is indicated for single vitrified-warmed blastocyst transfer, the blastocyst will be warmed on the embryo transfer day using the Cryotop warming method (Vitrolife, Sweden).

Post-warming blastocyst culture and blastocyst transfer procedure and luteal support

Embryos were cultured in the continuous single culture NXC plate (Irvine Scientific) after warming for at least 1 h before embryo transfer. Blastocyst quality was assessed before transferring. Only blastocysts that maintained the same quality or developed better compared to blastocyst quality before vitrification were used in this study. A decreased-quality blastocyst will not be included in this study.

The hormonal replacement therapy (HRT) protocol was initiated on the second day of

the menstrual cycle for all patients. To ensure optimal endometrial thickness, patients were required to undergo ultrasound check-ups of the endometrium, uterus, and adnexa on days 10 and 14 of the menstrual cycle. Once the endometrial thickness reached a minimum of 7 mm, endometrial transformation was achieved using vaginal micronized progesterone at a daily dosage of 600–800 mg (Utrogestan, Besins, Thailand or Cyclogest, Actavis U.K. Limited, United Kingdom), in addition to orally administered dydrogesterone at a daily dose of 30 mg (Duphaston, Abbott Biologicals B.V., Netherlands). Blastocyst embryos were transferred after approximately 120 ± 3 h. The embryo transfer procedure adhered to the 2017 guidelines by the American Society for Reproductive Medicine (ASRM). In situations where the catheter encountered difficulty passing through the cervical canal into the uterine cavity, resulting in prolonged transfer time and requiring the use of a catheter with a malleable mandrel or cervical clamp, the procedure was recorded as a difficult embryo transfer.

Pregnancy outcomes

For clinical outcomes, we used the following definitions: pregnancy was confirmed (serum hCG > 5 mIU/mL); implantation was confirmed at 8 weeks of pregnancy by observation of the gestational sac by ultrasound; clinical pregnancy, with a confirmed gestational sac at 6–7 weeks of pregnancy; ongoing pregnancy: defined as pregnancy that continued beyond 12 weeks of gestation.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences 26 (SPSS Inc.).

To describe the data, descriptive statistics, frequency analysis, and percentage analysis were used for categorical variables, and the mean and standard deviation (mean ± SD) were used for continuous variables. Statistical tests for quantitative variables, when comparing the average value in two research groups, use the ANOVA test. Statistical tests for qualitative variables, when comparing two proportions, use the Chi-square test.

Using the GEEs to evaluate the relationship between the EEVA system, blastocyst morphological assessment, or a combination of the EEVA system and blastocyst morphological assessment, and implantation and ongoing pregnancy. Comparison of 3 methods to evaluate the results of predicting pregnancy outcomes using the area under the Receiver operating characteristic (ROC) curve (AUC): performed on the prediction probability scale of the model rule. The GEE model for pregnancy outcomes included maternal age, BMI of the oocyte provider, and the day of embryo transfer (Day 5 vs Day 6) as confounders. The GEE model considers multiple embryos from a single patient as an intra-subject variable, standardizing patient-related confounders.

The adjusted odds ratios (aORs) of the effect of the variables included in the GEEs on the outcome variables were expressed as 95% CI, and statistical significance was considered for *P*-values < 0.05. The ROC curves were graphed from the probability values obtained by the GEE models, and the AUC was calculated as a model evaluation metric. A comparison of AUCs was performed using a paired, two-tailed DeLong's test. The 2 AUCs were statistically significantly different when $p < 0.05$.

Ethics statement

This study was approved by the Internal Committee for Ethics of the Tam Anh General Hospital, Hanoi, Vietnam (approval number: IRB.TAHN.055) and conducted according to the Good Clinical Practice and the Declaration of Helsinki 2002 principles.

RESULTS

Demographic and clinical characteristics of patients

We analyzed the pregnancy outcomes of 511 single vitrified-warmed blastocyst transfer cycles from 2020 to 2023. A general description of the study population, including cycle, demographic, and embryonic characteristics, was presented by the parameters in Table 1.

Table 1. General descriptive and demographic characteristics of the cycles.

Characteristics	Values
Cycles (n)	511
Maternal age (years)	30.5 ± 4.9
Endometrial thickness (mm)	9.5 ± 1.2
BMI (kg/m^2)	21.07 ± 2.54
Embryo Day 5	90% (460/511)
Embryo Day 6	10% (51/511)
Blastocoel expansion (BL) degree	
BL3	3.91% (20/511)
BL4	10.37% (53/511)
BL5	47.55% (243/511)
BL6	31.51% (161/511)
BL7	6.66% (34/511)
Inner cell mass (ICM) grade	
ICM A	68.88% (352/511)
ICM B	22.12% (113/511)
ICM C	9% (46/511)
Trophectoderm (TE) grade	
TE A	61.06% (312/511)
TE B	30.71% (157/511)
TE C	8.23% (42/511)
EEVA score of blastocyst transfer	
E1	43.05% (220/511)
E2	26.22% (134/511)
E3	14.87% (76/511)
E4	10.18% (52/511)
E5	5.68% (29/511)
Implantation rate (%)	67.1%
Clinical pregnancy rate (%)	54.6%
Ongoing pregnancy rate (%)	49.7%
Live birth rate (%)	45%

Continuous data are expressed as mean ± SD; categorical data are expressed as percentage (%). BMI: BMI of the oocyte provider. EEVA: Early embryo viability assessment, E: EEVA score.

No differences were found in the maternal age, endometrial thickness, BMI, or embryo expansion degree between the implantation and non-implantation groups, the ongoing pregnancy and non-ongoing pregnancy groups ($p > 0.05$). This suggests that in the study population, these factors were not direct determinants of pregnancy outcomes. The rates of Day 5 blastocysts, ICM A, TE A, and E1 were significantly higher in the implantation and ongoing pregnancy groups compared with the non-implantation and non-ongoing pregnancy groups, respectively ($p < 0.05$), as detailed in Table 2. Day 5 blastocysts have a higher chance of implantation and ongoing pregnancy than Day 6 blastocysts. ICM A, TE A quality and EEVA 1 score are strong predictors of implantation and ongoing pregnancy.

Table 2. Descriptive and demographic characteristics in implantation and non-implantation groups; ongoing pregnancy and non-ongoing pregnancy groups.

Characteristics	Implantation	Non-implantation	P value
Maternal age (years)	30.1 ± 4.2	30.9 ± 4.6	0.06
Endometrial thickness	9.5 ± 1.2	9.4 ± 1.4	0.456
BMI	21.1 ± 2.5	20.9 ± 2.5	0.44
Embryo Day 5	319 (93.0%)	141 (83.9%)	0.001
Embryo Day 6	24 (7.0%)	27 (16.1%)	
Blastocoel expansion (BL) degree			
BL3	12 (3.5%)	8 (4.8%)	
BL4	35 (10.2%)	18 (10.7%)	
BL5	167 (48.7%)	76 (45.2%)	0.588
BL6	110 (32.1%)	51 (30.4%)	
BL7	19 (5.5%)	15 (8.9%)	
Inner cell mass (ICM) grade			
ICM A	271 (79%)	81 (48.2%)	
ICM B	58 (16.9%)	55 (32.7%)	< 0.001
ICM C	14 (4.1%)	32 (19.0%)	
Trophectoderm (TE) grade			
TE A	248 (72.3%)	64 (38.1%)	
TE B	86 (25.1%)	71 (42.3%)	< 0.001
TE C	9 (2.6%)	33 (19.6)	
EEVA score of blastocyst transfer			
E1	169 (49.3%)	51 (30.4%)	
E2	97 (28.3%)	37 (22.0%)	
E3	40 (11.7%)	36 (21.4%)	< 0.001
E4	24 (7.0%)	28 (16.7%)	
E5	13 (3.8%)	16 (9.5%)	

	Ongoing pregnancy	**Non-ongoing pregnancy**	**P value**
Maternal age (years)	29.7 ± 4.05	30.9 ± 4.5	0.052
Endometrial thickness	9.6 ± 1.2	9.4 ± 1.3	0.059
BMI	21.1 ± 2.5	21.1 ± 2.5	0.953
Embryo Day 5	239 (94.1%)	221 (86.0%)	0.002
Embryo Day 6	15 (5.9%)	36 (14.0%)	
Blastocoel expansion degree			
BL3	8 (3.1%)	12 (4.7%)	
BL4	26 (10.2%)	27 (10.5%)	
BL5	121 (47.6%)	122 (47.5%)	0.669
BL6	85 (33.5%)	76 (29.6%)	
BL7	14 (5.5%)	20 (7.8%)	
Inner cell mass grade			
ICM A	213 (83.9%)	139 (54.1%)	
ICM B	35 (13.8%)	78 (30.4%)	< 0.001
ICM C	6 (2.4%)	40 (15.6%)	
Trophectoderm grade			
TE A	199 (78.3%)	113 (44%)	
TE B	52 (20.5%)	105 (40.9%)	< 0.001
TE C	3 (1.2%)	39 (15.2%)	
EEVA score of blastocyst transfer			
E1	135 (53.1%)	85 (33.1%)	
E2	73 (28.7%)	61 (23.7%)	
E3	25 (9.8%)	51 (19.8%)	< 0.001
E4	14 (5.5%)	38 (14.8%)	
E5	7 (2.8%)	22 (8.6%)	

Continuous data are expressed as mean ± SD; categorical data are expressed as percentage (%). BMI: BMI of the oocyte provider. EEVA: Early embryo viability assessment, E: EEVA score.

Association between univariate analysis and implantation and ongoing pregnancy

A GEEs model was built to quantify the odds of achieving implantation and ongoing pregnancy according to univariate factors, including maternal age, endometrial thickness, BMI, embryo age, embryo expansion, ICM morphology, TE morphology, and EEVA score. Embryo age, ICM morphology, TE morphology, and EEVA score significantly correlated with implantation and ongoing pregnancy ($p < 0.05$), as presented in Table 3. The aORs (Day 6 vs Day 5) were 0.393 (95% CI: 0.219–0.705, $p = 0.002$) for implantation and 0.385 (95% CI: 0.205–0.723, $p = 0.003$) for ongoing pregnancy. Day 6 blastocysts have approximately 61% lower implantation and ongoing pregnancy potential than Day 5

blastocysts. The aOR (ICM B vs ICM A) was 0.315 (95% CI: 0.202–0.492, p < 0.001) for implantation and 0.293 (95% CI: 0.186–0.460, p < 0.001) for ongoing pregnancy. Blastocysts with ICM B have approximately 68% and 71% lower implantation and ongoing pregnancy potential than blastocysts with ICM A. The aOR (ICM C vs ICM A) was 0.131 (95% CI: 0.067–0.257, p < 0.001) for implantation and 0.098 (95% CI: 0.040–0.237, p < 0.001) for ongoing pregnancy. Blastocysts with ICM C have approximately 86% and 90% lower implantation and ongoing pregnancy potential than blastocysts with ICM A. Similarly to TE quality, blastocysts with TE B have approximately 69% and 72% lower implantation and ongoing pregnancy potential than blastocysts with TE A. Blastocysts with TE C have approximately 93% and 95% lower implantation and ongoing pregnancy potential than blastocysts with TE A. Embryos with higher EEVA scores (E3, E4 and E5) have approximately 67%, 74% and 76% lower implantation potential than embryos with EEVA 1, respectively as well as approximately 69%, 77% and 80% lower ongoing pregnancy potential than embryos with EEVA 1.

Table 3. Generalized estimating equations assessing the association of univariate variables with implantation and ongoing pregnancy.

	Univariate analysis		
Characteristics	**aOR**	**95% CI**	**P value**
Implantation			
Maternal age (years)	-		0.069
Endometrial thickness	-		0.455
BMI	-		0.437
Embryo age (Day 6 vs Day 5)	0.393	0.219-0.705	0.002
Blastocoel expansion (BL) degree			
BL4 vs BL3	-		0.631
BL5 vs BL3	-		0.423
BL6 vs BL3	-		0.456
BL7 vs BL3	-		0.768
Inner cell mass (ICM) grade			
ICM B vs ICM A	0.315	0.202-0.492	< 0.001
ICM C vs ICM A	0.131	0.067-0.257	< 0.001
Trophectoderm (TE) grade			
TE B vs TE A	0.313	0.206-0.475	< 0.001
TE C vs TE A	0.070	0.032-0.155	< 0.001
EEVA score			
E2 vs E1	0.791	0.484-1.293	0.350
E3 vs E1	0.335	0.194-0.580	< 0.001
E4 vs E1	0.259	0.138-0.485	< 0.001

E5 vs E1	0.245	0.111-0.544	0.001
Ongoing pregnancy			
Maternal age (years)	0.938	0.899-0.977	0.052
Endometrial thickness	-		0.059
BMI	-		0.953
Embryo age (Day 6 vs Day 5)	0.385	0.205-0.723	0.003
Blastocoel expansion degree			
BL4 vs BL3	-		0.490
BL5 vs BL3	-		0.402
BL6 vs BL3	-		0.284
BL7 vs BL3	-		0.932
Inner cell mass grade			
ICM B vs ICM A	0.293	0.186-0.460	< 0.001
ICM C vs ICM A	0.098	0.040-0.237	< 0.001
Trophectoderm grade			
TE B vs TE A	0.281	0.188-0.421	< 0.001
TE C vs TE A	0.044	0.013-0.145	< 0.001
EEVA score			
E2 vs E1	0.753	0.488-1.164	0.202
E3 vs E1	0.309	0.178-0.535	< 0.001
E4 vs E1	0.232	0.119-0.453	< 0.001
E5 vs E1	0.200	0.082-0.489	< 0.001

BMI: BMI of the oocyte provider. EEVA: Early embryo viability assessment, E: EEVA score. aOR: adjusted odds ratio. CI: confidence interval. P < 0.05: statistical significance of the variable-outcome association.

Association between EEVA scores with implantation and ongoing pregnancy

The predictive power of the EEVA system classification in multivariate GEEs analyzed for implantation and ongoing pregnancy is shown in Table 4. Only variables in the univariate model that have statistically significant differences are included in the multivariate model. The EEVA score significantly correlated with implantation and ongoing pregnancy, in which the OR of each category was decreasingly lower. The ORs for implantation and ongoing pregnancy were significantly lower in E3, E4, and E5 when compared to E1 ($p < 0.05$), except those between E1 and E2 (Table 4). The embryonic age variable was not significantly different between implantation and non-implantation, ongoing pregnancy and non-ongoing pregnancy in the multivariate GEE models after adjusting for other factors ($p = 0.074$, $p = 0.1$) (Table 4).

Table 4. Generalized estimating equations assessing the association of the EEVA score with implantation and ongoing pregnancy, alongside embryo age.

	Multivariate analysis		
Characteristics	**aOR**	**95% CI**	**P value**
Implantation			
Embryo age (Day 6 vs Day 5)	-		0.074
EEVA score			
E2 vs E1	0.795	0.486-1.301	0.362
E3 vs E1	0.350	0.201-0.607	< 0.001
E4 vs E1	0.288	0.151-0.546	< 0.001
E5 vs E1	0.282	0.125-0.636	0.002
Ongoing pregnancy			
Embryo age (Day 6 vs Day 5)	-		0.1
EEVA score			
E2 vs E1	0.757	0.489-1.170	0.21
E3 vs E1	0.321	0.184-0.557	< 0.001
E4 vs E1	0.256	0.129-0.505	< 0.001
E5 vs E1	0.228	0.092-0.563	0.001

EEVA: Early embryo viability assessment, E: EEVA score. aOR: adjusted odds ratio. CI: confidence interval. P < 0.05: statistical significance of the variable-outcome association.

Association between blastocyst morphology with implantation and ongoing pregnancy

The predictive power of ICM and TE morphology test classification in multivariate GEEs analyzed for implantation and ongoing pregnancy is shown in Table 5. Only variables in the univariate model that have statistically significant differences are included in the multivariate model. TE morphology significantly correlated with implantation and ongoing pregnancy, in which the OR of each category was decreasingly lower. The ORs for implantation and ongoing pregnancy were significantly lower in TE B and TE C when compared to TE A ($p < 0.05$) (Table 5). The embryonic age variable and ICM morphology were not significantly different in the multivariate GEE model after adjusting for other factors ($p > 0.05$) (Table 5).

Table 5. Generalized estimating equations assessing the association of the morphology with implantation and ongoing pregnancy, alongside embryo age.

	Multivariate analysis		
Characteristics	**aOR**	**95% CI**	**P value**
Implantation			
Embryo age (Day 6 vs Day 5)	-		0.759
Inner cell mass (ICM) grade			
ICM B vs ICM A	-		0.073
ICM C vs ICM A	-		0.929
Trophectoderm (TE) grade			
TE B vs TE A	0.405	0.240-0.683	0.001
TE C vs TE A	0.072	0.017-0.293	< 0.001
Ongoing pregnancy			
Embryo age (Day 6 vs Day 5)	-		0.703
Inner cell mass grade			
ICM B vs ICM A	-		0.053
ICM C vs ICM A	-		0.614
Trophectoderm grade			
TE B vs TE A	0.371	0.226-0.611	< 0.001
TE C vs TE A	0.058	0.010-0.318	0.001

EEVA: Early embryo viability assessment, E: EEVA score. aOR: adjusted odds ratio. CI: confidence interval. P < 0.05: statistical significance of the variable-outcome association.

Association between the combination of EEVA scores and blastocyst morphology with implantation and ongoing pregnancy

The predictive power of the EEVA system classification combined with ICM, TE morphology, and embryo age in multivariate GEEs analyzed for implantation and ongoing pregnancy is shown in Table 6. Only variables in the univariate model that have statistically significant differences are included in the multivariate model. EEVA scores and TE morphology significantly correlated with implantation and ongoing pregnancy, in which the OR of each category was decreasingly lower. The ORs for implantation and ongoing pregnancy were significantly lower in TE B and TE C when compared to TE A ($p < 0.05$), and significantly lower in E3, E4, and E5 when compared to E1 ($p < 0.05$), except for those between E1 and E2 (Table 6). The embryonic age variable and ICM morphology were not significantly different in the multivariate GEE model after adjusting for other factors ($p > 0.05$) (Table 6).

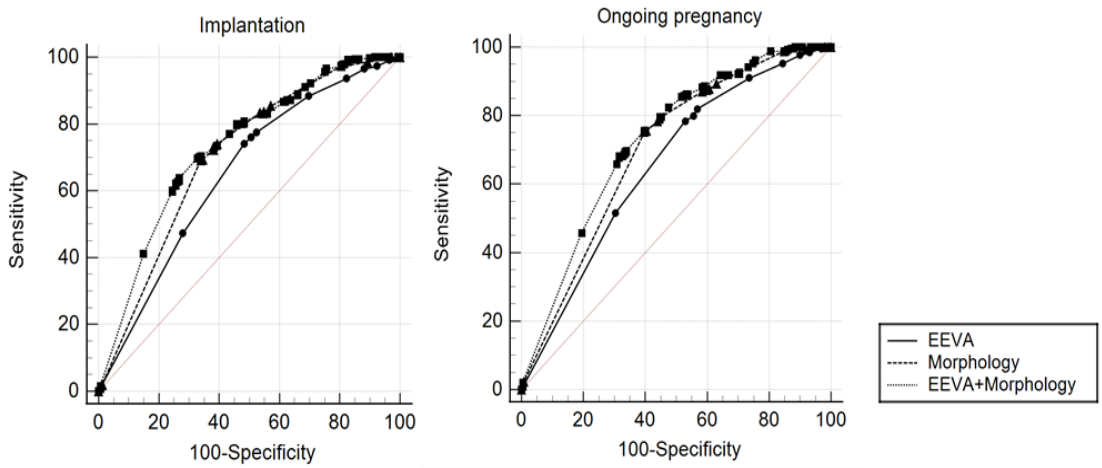
Table 6. Generalized estimating equations assessing the association of the EEVA scores and morphology together with implantation and ongoing pregnancy, alongside embryo age.

	Multivariate analysis		
Characteristics	**aOR**	**95% CI**	**P value**
Implantation			
Embryo age (Day 6 vs Day 5)	-		0.559
Inner cell mass (ICM) grade			
ICM B vs ICM A	-		0.214
ICM C vs ICM A	-		0.699
Trophectoderm (TE) grade			
TE B vs TE A	0.436	0.256-0.742	0.002
TE C vs TE A	0.065	0.016-0.267	< 0.001
EEVA score			
E2 vs E1	0.928	0.552-1.563	0.780
E3 vs E1	0.514	0.282-0.935	0.029
E4 vs E1	0.402	0.199-0.810	0.011
E5 vs E1	0.338	0.258-1.591	0.017
Ongoing pregnancy			
Embryo age (Day 6 vs Day 5)	-		0.473
Inner cell mass grade			
ICM B vs ICM A	-		0.194
ICM C vs ICM A	-		0.965
Trophectoderm grade			
TE B vs TE A	0.400	0.240-0.666	< 0.001
TE C vs TE A	0.052	0.010-0.288	0.001
EEVA score			
E2 vs E1	0.874	0.551-1.385	0.566
E3 vs E1	0.463	0.256-0.839	0.011
E4 vs E1	0.364	0.174-0.762	0.007
E5 vs E1	0.266	0.207-1.544	0.007

EEVA: Early embryo viability assessment, E: EEVA score. aOR: adjusted odds ratio. CI: confidence interval. P < 0.05: statistical significance of the variable-outcome association.

Comparison of three models

The ROC curves of three models are presented in Figure 2. The AUC of the model using the EEVA system only, blastocyst morphological assessment only, or a combination of the EEVA system and blastocyst morphological assessment was 0.651 (95% CI 0.608–0.692), 0.703 (95% CI 0.662–0.743), and 0.730 (95% CI 0.688–0.767), respectively, for implantation; and 0.655 (95% CI 0.612–0.696), 0.700 (95% CI 0.658–0.740), and 0.726 (95% CI 0.686–0.765), respectively, for ongoing pregnancy. The model with the highest AUC for implantation and ongoing pregnancy was always the one combining both the EEVA system and blastocyst morphological assessment when compared with models using only the EEVA system or blastocyst morphological assessment. The differences were statistically significant ($p < 0.05$). The difference between the model using only the EEVA system and blastocyst morphological assessment was not statistically significant. The dates are presented in Figure 2.



Model	AUC (95% CI)	
	Implantation	Ongoing pregnancy
EEVA	0.651 (0.608-0.692)	0.655 (0.612-0.696)
Morphology	0.703 (0.662-0.743)	0.700 (0.658-0.740)
EEVA + Morphology	0.730 (0.688-0.767)	0.726 (0.686-0.765)
	P value	
Pairwise comparison of ROC curves	Implantation	Ongoing pregnancy
EEVA vs Morphology	0.0633	0.0684
EEVA + Morphology vs EEVA	0.0001	0.0001
EEVA + Morphology vs Morphology	0.0378	0.0207

Figure 2. Performance metrics for the generalized estimating equations (GEEs) for implantation and ongoing pregnancy prediction. Receiver operating characteristic (ROC) curves and area under the ROC curves (AUCs) of the GEE are modeled for assessing the performance of the three classification systems: the EEVA system and blastocyst morphological assessment, as well as a combination of both, for implantation and ongoing pregnancy prediction. $P < 0.05$, statistical significance.

DISCUSSION

To our knowledge, this is the first study to test an embryo selection model that combines two components: (1) the EEVA system, an automatic classification algorithm applied to cleavage-stage embryos, and (2) the conventional blastocyst morphological assessment. The model was evaluated in the context of single vitrified-warmed blastocyst transfers, using implantation and ongoing pregnancy as the primary endpoints. The EEVA system can identify on the Day 3 embryos with the highest potential to reach the blastocyst stage. The results of our study confirm that the EEVA system is also useful in embryo selection on Days 5 and 6 for single vitrified-warmed blastocyst transfer, especially when the EEVA system is used in combination with the conventional blastocyst morphological assessment.

As for predicting the implantation potential of the EEVA system, this study showed that embryos with a low score of EEVA had a higher implantation rate than those with a high score of EEVA. There was a study that confirmed the predictive power of the EEVA system for implantation, in which embryos from oocyte donations with a low score of EEVA also had higher implantation rates than those with a higher score of EEVA; this was an external validation (Aparicio-Ruiz *et al*., 2016). Another study that confirmed the predictive power of the EEVA system for implantation (Valera *et al*., 2023). The OR of E1 vs E5 was 2.920 (95% CI 1.440–5.925; p = 0.003; E = 2.81) for implantation and 3.317 (95% CI 1.615–6.814; p = 0.001; E = 3.04) for live birth. However, in PGT-A cases, this association was not statistically significant differences (Valera *et al*., 2023). The results of our study showed the ability of the EEVA system to identify embryos with higher implantation potential. Transferred vitrified-warmed blastocysts with the high EEVA score (E5) had 0.282 times lower odds of successful implantation than blastocysts with the low EEVA score (E1), $p < 0.05$ (Table 4). Our study is also the first to test a single vitrified-warmed blastocyst transfer for ongoing pregnancy as the endpoint. Transferred vitrified-warmed blastocysts with the high EEVA score (E5) had 0.228 times lower odds of a successful ongoing pregnancy than blastocysts with the low EEVA score (E1), $p < 0.05$ (Table 4).

Prolonging the culture of the cleavage embryo stage to the blastocyst stage has now become a routine practice in many IVF laboratories. Consequently, new selection algorithms tend to use later morphokinetic events, or a combination of algorithms and morphology assessment (Carrasco *et al*., 2017). Among the algorithms for blastocyst selection, one study used two morphokinetic parameters: the synchrony in divisions of the third cell cycle (s3) and the time to reach the expanded blastocyst stage (tEB), with an AUC = 0.602 (95% CI 0.559–0.645), but it has not been externally validated (Motato *et al*., 2016). Another study used some parameters and the start of blastulation time (tSB) as a positively scoring variable (Goodman *et al*., 2016). However, this model did not mention AUC. An external validation of another predictive algorithm, KIDScore D5 version 3, used for the selection of embryos at the blastocyst stage, available for EmbryoScope and EmbryoScope Plus incubators, with an AUC = 0.633 for implantation (Bori *et al*., 2020). The KIDScore D5 was designed as an embryo classification system independent of the conventional blastocyst morphological assessment, as it already includes

trophectoderm and inner cell mass morphology as predictive variables in the algorithm. Contrary to KIDScore D5, the EEVA system was developed as a complementary tool to the conventional morphological evaluation of day 3 embryos to predict blastocyst formation (Conaghan *et al*., 2013).

Regarding blastocyst morphology, several recent studies using artificial intelligence-based embryo assessment systems, including the EEVA system and other time-lapse platforms, have shown that TE quality is a stronger predictor of implantation and ongoing pregnancy outcomes than ICM and blastocyst expansion stage (Bartolacci *et al*., 2024; Chen *et al*., 2014). Multivariate regression analysis results in many reports showed that, after adjusting for confounding factors, TE still plays an independent and strong prognostic role for implantation, while the effects of ICM and blastocyst expansion become insignificant ($p > 0.05$) (He *et al*., 2021; Hill *et al*., 2013). This reinforces the hypothesis that AI models such as EEVA, when integrating TE morphological data, can significantly improve the accuracy of embryo selection for transfer, especially in the context of single blastocyst transfer. Our study showed that both implantation and ongoing pregnancy odds ratios were significantly reduced for TE grades B and C compared with TE grade A ($p < 0.05$). In multivariate GEE models, including EEVA, ICM, TE morphology and embryo age variables, TE morphology still plays an independent and strong prediction role for implantation and ongoing pregnancy (Table 6). Depending on the type of embryo transfer cycle, many embryo selection algorithms based on automatic annotations have been reported to achieve AUCs of approximately 0.650–0.700, achieving a statistically significant ability to predict implantation, but only at a relatively high level. None of these models showed a statistically significant improvement compared to blastocyst morphological assessment (Valera *et al*., 2023). In a study, EEVA classification performed similarly to traditional blastocyst morphological assessment for implantation prediction. The combination of the EEVA system and the traditional blastocyst morphological assessment tended to have better prognostic value than EEVA classification alone, AUCs = 0.636 (95% CI 0.584-0.660); 0.622 (95% CI 0.598-0.673), respectively. However, the difference was not statistically significant ($p > 0.05$) (Valera *et al*., 2023).

In our study, the EEVA classification also performed similarly to the traditional blastocyst morphological assessment for implantation prediction with an AUC = 0.651 (95% CI 0.608–0.692) vs 0.703 (95% CI 0.662–0.743), respectively, and for ongoing pregnancy prediction with an AUC = 0.655 (95% CI 0.612-0.696) vs 0.700 (95% CI 0.658–0.740), respectively. The differences were not statistically significant ($p > 0.05$) (Figure 2). Further analysis of the GEE model using a combination of both methods, including the EEVA system and the blastocyst morphological assessment, showed that both predictors were statistically significant for implantation and ongoing pregnancy prediction, and their predictive values were independent of each other. Indeed, in this study, the best AUCs for both outcomes were found when combining both the evaluation systems of the EEVA system and the blastocyst morphological assessment compared with the EEVA system alone or the blastocyst morphological assessment alone: the AUC =

0.730 (95% CI 0.688-0.767), 0.651 (95% CI 0.608-0.692), and 0.703 (95% CI 0.662-0.743) for implantation, respectively; the AUC = 0.726 (95% CI 0.686-0.765), 0.655 (95% CI 0.612-0.696) and 0.700 (95% CI 0.658-0.740) for ongoing pregnancy, respectively. The differences in pairwise comparison of ROC curves were statistically significant, $p < 0.05$ (Figure 2).

Our study used GEEs for statistical analysis. GEEs consider several events as intra-patient variables. This way, the model considers the relationship between embryos that share the same patient background characteristics, adding one more layer to the consideration of the intra-data context of measured confounders. We only selected blastocysts whose quality did not decrease after warming. Therefore, the vitrification procedure did not adversely affect the quality of the transferred embryo. In this study, we analyzed univariate data using the GEE model; only statistically significant variables were included in the multivariate model. The age of the transferred embryo was included in the three GEE models using the EEVA system only, blastocyst morphological assessment only, and a combination of both. However, in all 3 models, the age of the transferred embryo was not statistically significant when combined with the variables ($p > 0.05$).

The limitation of the study was that the details of the algorithm and some morphological features used in the EEVA system were hidden by the manufacturer, which prevented our later conclusions because we did not know how to calculate the score. The EEVA score is automatically obtained from the software. The performance of automatic annotations of morphokinetic event timings provided by the EEVA system may be unclear. In this study, the automatic annotations were not validated, as we aimed to analyze the performance of the whole system. In addition, it should be noted that our study was retrospective and therefore may have limitations. Therefore, in future studies, randomized controlled trials may be required.

In conclusion, our results confirm the efficacy of the EEVA algorithm for implantation and ongoing pregnancy outcome prediction. The highest AUC was achieved when combining the EEVA system and blastocyst morphological assessment, and the improvement over the EEVA system alone or morphological assessment alone was statistically significant.

ACKNOWLEDGMENTS

The authors would like to thank the members of the IVF laboratory Tam Anh for supporting all the lab's work during the group's research. Specifically, we would like to thank Tam Anh General Hospital for allowing and facilitating us to complete the study. We would like to thank the Graduate University of Science and Technology, Vietnam Academy of Science and Technology, Hanoi, Vietnam for approving the research.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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