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ORIGINAL ARTICLE *Embryology*

Prediction of live birth in frozen–thawed single blastocyst transfer cycles by pre-freeze and post-thaw morphology

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STUDY QUESTION: What pre-freeze and post-thaw morphological parameters can be used to predict live birth outcomes after frozen–thawed blastocyst transfer cycles?

SUMMARY ANSWER: Pre-freeze blastocoele expansion and trophectoderm (TE) grade and post-thaw degree of re-expansion are the most significant predictors of live birth in frozen–thawed blastocyst transfer cycles.

WHAT IS KNOWN ALREADY: Currently, blastocoele re-expansion after thawing is used to indicate blastocyst cryosurvival and reproductive potential. The predictive roles of other pre-freeze and post-thaw morphological parameters are neglected.

STUDY DESIGN, SIZE, DURATION: This was a retrospective study of all the patients who received a frozen–thawed single blastocyst transfer ($n = 1089$) at our clinic between March 2008 and October 2011.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Pre-freeze morphological parameters analyzed for all blastocysts included grade of blastocoele expansion, inner cell mass and TE. A group of blastocysts ($n = 243$) were also graded for post-thaw parameters: degree of blastocoele re-expansion, viability and cell contour. Univariate and multivariate generalized estimating equations (GEEs) models were used to identify the confounders that statistically significantly affected live birth outcomes and to investigate the independent effect of significant pre-freeze and post-thaw morphological parameters. Stepwise logistic regression analysis was used to select the best independent morphological predictors of live birth. Pearson correlations and linear regression analyses were performed to determine the relationship between morphological parameters and possible covariates.

MAIN RESULTS AND THE ROLE OF CHANCE: Multivariate GEE models estimated that the odds of live birth increased by ~36% for each grade of expansion ($P = 0.0061$) and decreased by 29% for blastocysts with grade B TE compared with grade A TE ($P = 0.0099$). Furthermore, the odds of live birth increased by ~39% ($P = 0.0042$) for each 10% increase in degree of re-expansion. Blastocoele expansion and TE grade were selected as the most significant pre-freeze morphological predictors of live birth and degree of re-expansion was selected as the best post-thaw parameter for prediction of live birth.

LIMITATIONS, REASONS FOR CAUTION: Blastocysts with poorer grades of morphology were not cryopreserved or transferred, limiting the ability to generalize our findings for grades of morphology not included in this study.

WIDER IMPLICATIONS OF THE FINDINGS: Blastocysts with higher pre-freeze grades of expansion and TE, irrespective of day of cryopreservation, should be given priority when thawing. Subsequently, re-expanding blastocysts, assessed within 2–4 h, with >60% viability should be transferred.

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TRIAL REGISTRATION NUMBER: not applicable.

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Introduction

Over the past decade vitrification has proved to be a successful method of blastocyst cryopreservation in human IVF, achieving high pregnancy and live birth rates (Mukaida *et al.*, 2003a; Stehlik *et al.*, 2005; Liebermann and Tucker, 2006). This method combines rapid cooling rates and high cryoprotectant concentrations to invoke a glass-like solid phase and minimize cellular injuries due to ice crystal formation. Further improvements, such as artificial collapse of the blastocoele and use of minute volume holding devices (e.g. electron microscopic grid and Cryoloop), have greatly improved the survival rates of vitrified blastocysts (Vanderzwalmen *et al.*, 2002; Mukaida *et al.*, 2003a,b). Importantly, our group has recently reported, in support of other studies, that obstetric outcomes of vitrified blastocysts do not differ from those of fresh blastocysts and blastocysts cryopreserved by slow-freeze methods (Takahashi *et al.*, 2005; Liebermann, 2009; Wikland *et al.*, 2010).

During vitrification and warming, blastocysts endure a number of morphological challenges, including blastocoele collapse followed by cell dehydration and then rehydration. These induced morphological changes can lead to cell damage and loss, disrupting morphological integrity. At present, morphology is an important tool used to predict embryo viability and in fresh IVF cycles three morphological parameters, degree of blastocoele expansion, inner cell mass (ICM) and trophectoderm (TE) cells, are part of an established grading system (Gardner and Schoolcraft, 1999; Gardner *et al.*, 2000; Alpha Scientists in Reproductive Medicine *et al.*, 2011). However, the predictive power of morphology once blastocysts are vitrified and warmed is not as clear. Currently, many IVF clinics use either the ability of the blastocoele to re-expand after warming and/or the pre-freeze score as good indicators of survival and reproductive potential (Shu *et al.*, 2009; Goto *et al.*, 2011; Honnma *et al.*, 2012).

No one grading system has been proposed and the importance of each parameter graded has not been elucidated. Therefore, the aims of this study were to determine whether (i) pre-freeze morphology, (ii) a three part post-thaw scoring system and (iii) combination of pre-free and post-thaw morphological parameters could be used to predict live birth outcomes after frozen–thawed blastocyst transfer cycles. Post-thaw criteria included degree of blastocoel re-expansion, degree of cell survival and degree of cell contour. The relationships between pre-freeze and post-thaw morphological parameters were also investigated.

Materials and Methods

Patients, stimulation protocol, IVF and embryo culture

In this study, a retrospective analysis of all the patients who received a frozen–thawed single blastocyst transfer at our clinic from March 2008 through to October 2011 was performed. Approval by the ethical committee at the University of Gothenburg was given for this study.

All the frozen–thawed blastocysts used in this study were obtained in stimulated oocyte retrieval cycles as previously described (Ahlstrom *et al.*, 2011). Briefly, all the patients were down-regulated with either GnRH agonists (Suprecur®, Hoechst, Germany) or GnRH antagonists (Olgalutran®, MSD, USA) and ovarian stimulation was achieved with recombinant FSH (Gonal-F® or Puregon®, Merck Serono, Germany) or highly purified hMG (Menopur, Ferring, Switzerland). Follicular aspiration was performed using vaginal ultrasonography 36–38 h after hCG (Ovitrelle, Merck Serono, Germany) administration. Retrieved oocytes were rinsed and placed in Cook fertilization media (Cook Medical, Australia) prior to insemination or injection, following standard techniques. After 16–18 h, fertilization was confirmed by the presence of two pronuclei and zygotes were placed into individual droplets of 25 μl of Cook cleavage media for culture. Patients with five or more zygotes are allegeable to receive blastocyst culture and Day 5 transfer. On Day 2, embryos were transferred into individual droplets of CCM^{TM} medium (Vitrolife AB, Sweden) for culture to the blastocyst stage. Blastocysts were graded on Days 5 and 6 of culture according to Gardner and Schoolcraft's system (Gardner and Schoolcraft, 1999). On Day 5, an embryo of grade ≥3BB was considered a good-quality embryo (GQE) and, if not transferred, cryopreserved by vitrification. On Day 6, the same criteria (≥3BB) were used to select blastocysts for cryopreservation.

Blastocyst vitrification and thawing

Blastocysts were vitrified and warmed according to the method first described by Lane *et al.* (1999) and later adapted by Mukaida *et al.* (2003a,b) to include artificial collapsing of the blastocyst with a laser (Fertilase MTG, Altdorf, Germany) (Hardarson *et al.*, 2007). A 1.48 diode laser is used to breach the zona pellucida and TE cells ~15 min before initiating the vitrification process (Lane *et al.*, 1999; Mukaida *et al.*, 2003a; Wikland *et al.*, 2010). Vitrification and warming solutions were provided in the VitriBlast kit and the ThermoBlast kit (Nidacon, Gothenburg, Sweden). All the solutions were incubated at 37°C for 30 min prior to vitrification and warming, and all the steps were carried out on heated stages. For vitrification blastocysts were first equilibrated in solution I containing HEPES/bicarbonate-based base medium for 2 min and then placed into vitrification solution II containing 7.5% dimethyl sulphoxide (DMSO) and 7.5% ethylene glycol for 2 min before being transferred to vitrification solution III, which contains 15% DMSO and 15% ethylene glycol, 12 mM ficoll and 0.58 M sucrose, for a minimum of 30 s. Each blastocyst was then loaded onto a cryoloop (Vitrolife), plunged into liquid nitrogen and then capped with a cryovial before being stored in standard cryotanks. This device is an open system. To warm, the cryoloop containing the blastocyst was submerged into the first thaw solution containing base medium and 0.28 M sucrose for 2 min, then moved to a second thaw solution containing base medium and 0.17 M sucrose for 3 min and finally washed once and incubated for 5 min in base medium without sucrose. After warming, blastocysts were cultured in CCM (Vitrolife) medium for up to 6 h before a single observation and assessment was made under an inverted microscope (×200 magnification) for a short interval (20–30 s) and the time recorded (time between warming and blastocyst assessment).

Post-thaw scoring of blastocysts

Blastocysts of the subgroup population were subjectively graded for each post-thaw parameter, degree of blastocoele re-expansion, degree of cell reorganization and degree of cell survival, according to a scale from 0 to 100% at 10% intervals. The time between warming and blastocyst assessment was then recorded. Only one morphological assessment was performed. Degree of re-expansion was determined by the size of the blastocoele and space between the TE and zona (Fig. 1). Degree of viability was determined by estimating the loss of blastomeres and the presence of dark cells and necrotic foci (Fig. 1). Grading cell contour was dependent on the ability to observe a discernible ICM and a cohesive TE cell layer (Fig. 1) with clear cellular boundaries. Previously, we used larger intervals of 100, 75, 66, 50, 33, 25 and 0%, but we marked that there was a tendency to grade up and down the different parameters and decided to

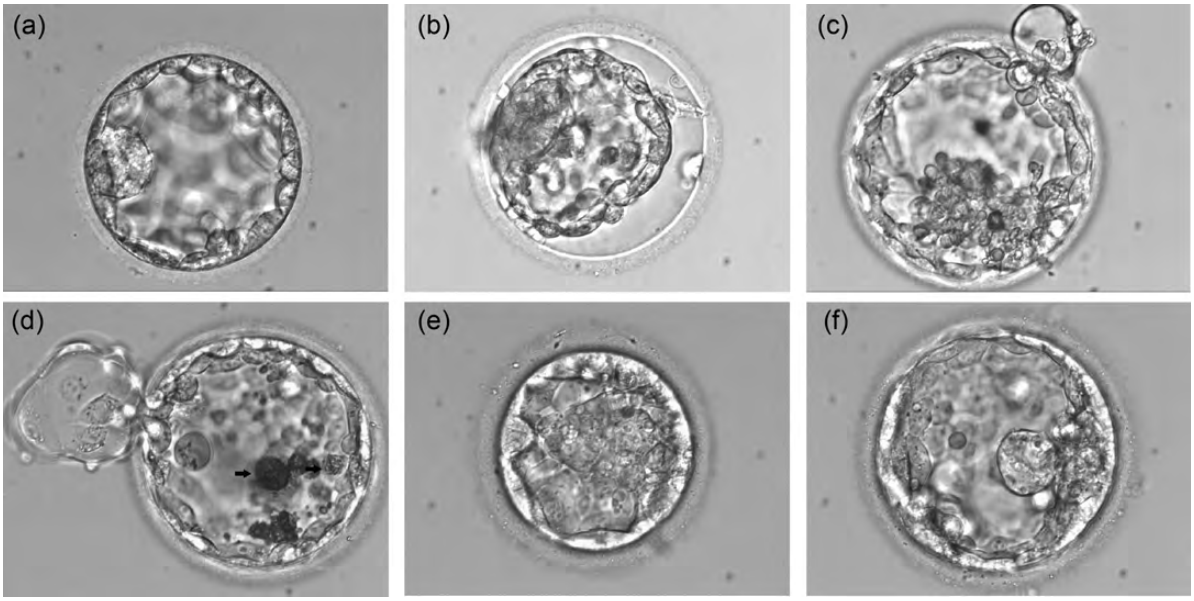

Figure 1 Photographic examples of scores for each post-thaw parameter. For grades of re-expansion 100% can be seen in (**a**) and 60% in (**b**). 100% degree of viability can be seen in (a, b and **e**); 90% in (**c** and **f**); and 80% in (**d**). Arrows in (d) indicate examples of dark non-viable cells and necrotic foci. Examples of 100% of degree of cell contour are shown in (a and b), as both blastocysts have compact ICM and elliptic TE cells with clear cell definition. Cell contour of 90% can be seen in (c) due to a slight reduction in compactness of the ICM and the presence of cell fragments. Blastocyst with 80% cell contour, (e), demonstrating that not all TE cells are ellipse and cell membranes are diffuse, such that the distinction between the two compartments, ICM and TE, is not clear. 60% cell contour in (f); ICM has disassembled and boundaries between individual TE cells are lost in many cells, as well as ellipsical shape (ICM, inner cell mass; TE, trophectoderm).

reduce the intervals to 10%. We also recognize that grading of morphology is a subjective method and variation can exist between embryologists. To reduce this variation, two embryologists graded each blastocyst.

Frozen–thawed blastocyst transfer cycle

Frozen–thawed blastocyst transfer cycles were performed as previously described by Wikland *et al.* (2010). The majority of warmed blastocysts were transferred in natural cycles 6 days after a positive urinary LH test. The remaining blastocysts were transferred in hormonally supplemented cycles. In these cycles patients received 6 mg of estradiol valerate (Progynon, Schering, Nordiska AB, Järfälla, Sweden) daily from cycle Day 1 to cycle Day 12–14. Endometrial thickness and texture was checked by vaginal ultrasound, and when the endometrium was at least 8 mm, patients began administration of micronized progesterone, 1200 mg daily for 6 days (vaginal suppositories, Progesterone MIC vag, Apoteketsbolaget AB, Umeå, Sweden), whereupon embryo transfer was performed. Estradiol and progesterone supplementation were continued for the following 2 weeks up to the pregnancy test and, if tested positive, for another 4–5 weeks.

Clinical outcome

The primary end-point was live birth outcome after frozen–thawed single blastocyst transfer. The live birth rate was defined as the number of live births out of the total number of transferred blastocysts.

Statistical analysis

All the statistical analyses were performed by using SAS statistical analysis program (SAS, USA).

Continuous variables are described with mean, SD and range, while categorical variables are described with *n* and %. For prediction of the clinical outcome live birth, generalized estimating equation (GEE) models were used as they allow adjustment of within-individual and within-hormonal treatment correlation. Univariate GEE models were used to identify the confounders that statistically significantly affect the outcome. The selected confounders were then included in a multivariable GEE analysis when investigating the effect of pre-freeze and then post-thaw morphological parameters.

Stepwise logistic regression was used for selection of independent statistically significant predictors among the morphology variables and the confounders. Once variables were selected, the GEE models were performed including the selected variables to obtain the adjusted odds ratios (ORs), 95% confidence intervals (CI) and associated *P*-values.

Pearson correlations, GEE and linear regression models were performed to determine the relationships between morphological parameters and possible covariates.

All significance tests were two-tailed and conducted at the 0.05 significance level.

Results

During the study period, 1111 frozen–thawed blastocyst transfers were performed. In total, 1303 blastocysts were warmed and 1133 (87%) were considered viable and transferred. Of these, 1089 (96.1%) were single blastocyst transfers (714 patients) and hence included in this study. The live birth rate was 38.9%. Characteristics of patients and cycles from which the frozen blastocysts were obtained are summarized and separated by live birth outcome in Table I, as are

Table I Patient and morphology characteristics of transferred blastocysts divided by live birth outcome.

Variable	No live birth (n = 665)	Live birth (n = 424)
Female age	36.5 ± 4.5 (23.0; 45.0)	35.2 ± 4.1 (23.0; 45.0)
BMI (kg/m^2)	23.8 ± 4.1 (16.5; 39.3) n = 446	23.2 ± 3.6 (16.5; 37.2) n = 253
Indication for cause of infertility		
Male	28.6% (190)	36.1% (153)
Female	30.5% (203)	28.1% (119)
Unexplained	40.9% (272)	35.8% (152)
No. of earlier cycles	0.73 ± 1.05 (0.0; 5.0)	0.63 ± 1.00 (0.0; 7.0)
FSH total dose	2022 ± 814 (650; 4950)	1908 ± 771 (725; 4950)
Agonist/antagonist		
Antagonist	58.9% (392)	58.5% (248)
Agonist	41.1% (273)	41.5% (176)
Follicles	16.6 ± 7.4 (3.0; 45.0)	16.0 ± 7.2 (3.0; 58.0)
Aspirated oocytes	11.7 ± 4.9 (2.0; 36.0)	11.5 ± 5.0 (2.0; 36.0)
Method		
Standard IVF	63.3% (421)	53.1% (225)
Microinjection	36.7% (244)	46.9% (199)
No. good-quality embryos	3.9 ± 1.9 (1.0; 12.0)	3.8 ± 1.9 (1.0; 14.0)
Day of freeze		
5	64.1% (426)	65.1% (276)
6	35.9% (239)	34.9% (148)
Expansion pre-freeze	3.6 ± 0.6 (2.0; 6.0)	3.7 ± 0.6 (2.0; 6.0)
Expansion pre-freeze		
2	0.8% (5)	1.9% (8)
3	42.6% (283)	30.4% (129)
4	54.9% (365)	65.1% (276)
5	1.4% (9)	2.4% (10)
6	0.5% (3)	0.2% (1)
Inner cell mass		
A	66.5% (439)	69.2% (288)
B	33.3% (220)	30.8% (128)
C	0.2% (1)	0.0% (0)
Trophectoderm cells		
A	37.3% (246)	47.1% (196)
B	62.6% (413)	52.9% (220)
C	0.2% (1)	0.0% (0)
No score for ICM and TC	5	8

For categorical variables % (n) is presented and for continuous variables mean ± SD (min; max) is presented. As not all patients had a recorded BMI in the database, the number is also provided.

the pre-freeze scores for blastocoele expansion, ICM and TE. The majority of blastocysts cryopreserved were of expansion grade 3 (37.8%) and 4 (58.9%) with grade A ICM (66.8%) and grade B TE (58.1%). A greater proportion of blastocysts were cryopreserved on Day 5 of development (64.5%). During the same study period, 9653 zygotes were cultured to blastocyst stage for Day 5 transfer and 34.7% developed to good-quality blastocysts and were either transferred on Day 5 or cryopreserved (Day 5 or 6).

Pre-freeze blastocyst morphology and prediction of live birth

Univariate analysis of patient and cycle variables showed that the likelihood of live birth decreased with each year of female age (OR 0.93, 95% CI 0.90–0.96, $P < 0.0001$) and for each 500th IU of FSH total dose used in the fresh IVF cycle (OR 0.90, CI 0.83–0.98, $P = 0.011$). Interestingly, the likelihood of live birth increased with

Table II Pre-freeze graded blastocysts: univariable GEE models for prediction of live birth.

Variable	Value	Live birth % (*n*)	Odds ratio (95% CI)	*P*-value
Female age	≤36.0	45.6% (256)		
	>36.0	31.9% (168)	0.93 (0.90–0.96)	<0.0001
BMI (kg/m^2)	≤22.8	38.1% (134)		
	>22.8	34.3% (119)	0.96 (0.92–1.0)	0.069
Indication for cause of infertility	Male	44.6% (153)	1.34 (0.97–1.85)	0.079
	Female	37.0% (119)		
	Unexplained	35.8% (152)	0.94 (0.69–1.29)	0.72
No. of earlier cycles	≤0.0	40.3% (258)		
	>0.0	37.0% (166)	0.91 (0.80–1.04)	0.16
FSH total dose (div by 500 mg)	≤3.6	42.6% (249)		
	>3.6	34.7% (175)	0.90 (0.83–0.98)	0.011
Agonist/antagonist	Antagonist	38.8% (248))		
	Agonist	39.2% (176	1.00 (0.78–1.30)	0.98
Follicle no.	≤15.0	41.9% (246)		
	>15.0	35.5% (178)	1.00 (0.98–1.01)	0.65
Aspirated oocyte no.	≤11.0	40.8% (241)		
	>11.0	36.7% (183)	1.00 (0.97–1.03)	0.98
Method	Standard IVF	34.8% (225		
	Microinjection	44.9% (199))	1.47 (1.15–1.90)	0.0026
No. of good-quality embryo	≤3.0	39.2% (218)		
	>3.0	38.6% (206)	1.03 (0.96–1.10)	0.46
Day of freeze	5	39.3% (276)		
	6	38.2 (148)	0.95 (0.74–1.22)	0.67
Expansion	2	61.5% (8)		
	3	31.3% (129)		
	4	43.1% (276)		
	5	52.6% (10)		
	6	25.0% (1)	1.38 (1.11–1.72)	0.0041
Inner cell mass	A	39.6% (288)		
	B	36.8% (128)	0.90 (0.69–1.16)	0.41
Trophectoderm cells	A	44.3% (196)		
	B	34.8% (220)	0.68 (0.53–0.87)	0.0020

Live birth rate (%) is stated for patients with the variable value. The total number of patients with each variable value can be calculated by dividing 100% by the live birth rate stated (%) and then multiplying it by (*n*).
For descriptive purposes, continuous variables have been dichotomized to less than or equal to the median value and greater than the median value, but original continuous variables were analyzed in the GEE model.
FSH total dose has been divided by 500 to facilitate interpretation of the OR (95% CI).
GEE models have been used as they allow adjustment of within-individual correlation.
For indication for causes of infertility, ORs for male versus female and unexplained versus female are presented.

use of ICSI compared with standard insemination (OR 1.47, 95% CI 1.15–1.90, $P = 0.0026$) (Table II).

The likelihood of live birth was not significantly affected by BMI, cause of infertility, number of earlier cycles, type of pituitary regulation (antagonist or agonist) number of follicles, number of aspirated oocytes, number of GQEs and day of blastocyst cryopreservation (Day 5 or 6).

From pre-freeze morphology we found that the likelihood of live birth significantly increased for each grade of expansion (OR 1.38, CI 1.11–1.72, $P = 0.0041$) and the odds of live birth were significantly lower for blastocysts of grade B TE compared with grade A TE (OR 0.68, CI 0.53–0.87, $P = 0.0020$). Pre-freeze ICM morphology did not significantly predict live birth. However, ICM was found to be the only pre-freeze parameter to significantly predict the odds of being transferred, as for each increase of ICM grade the odds of embryo transfer significantly increased (OR 0.68, CI 0.48–0.95, $P = 0.0253$).

Multivariate analyses using GEE models were performed to investigate the separate predictive effect of grade of expansion and grade of TE on live birth when adjusted for significant confounders, including female age and fertilization method. The odds of live birth increased by ~36% for each grade of expansion (OR 1.36, CI 1.09–1.70, $P = 0.0061$). The odds of live birth decreased by 29% for blastocysts with grade B TE compared with grade A TE (OR 0.71, CI 0.55–0.92, $P = 0.0099$).

To determine the best independent predictors of live birth, a stepwise logistic regression was performed using all statistically significant variables. This analysis selected female age, pre-freeze expansion grade, method of fertilization and pre-freeze TE grade, ordered from highest to lowest predictive strength, respectively.

Table III Post-thaw blastocyst parameters separated by live birth outcome.

Variable	No live birth (n = 131)	Live birth (n = 112)
Degree of re-expansion (%)	88.5 ± 14.0 (30; 100)	93.1 ± 9.7 (50; 100)
30	0.8% (1)	0.0% (0)
40	0.8% (1)	0.0% (0)
50	0.8% (1)	0.9% (1)
60	3.8% (5)	0.0% (0)
70	9.9% (13)	4.5% (5)
80	18.3% (24)	13.4% (15)
90	19.1% (25)	24.1% (27)
100	46.6% (61)	57.1% (64)
Degree of viable cells (%)	88.5 ± 8.8 (70; 100)	89.7 ± 9.6 (60; 100)
60	0.0% (0)	2.7% (3)
70	5.3% (7)	4.5% (5)
80	30.5% (40)	17.9% (20)
90	37.4% (49)	42.9% (48)
100	26.7% (35)	32.1% (36)
Degree of cell contour (%)	84.1 ± 10.6 (60; 100)	87.2 ± 10.8 (60; 100)
60	3.1% (4)	1.8% (2)
70	16.8% (22)	12.5% (14)
80	33.6% (44)	27.7% (31)
90	29.0% (38)	27.7% (31)
100	17.6% (23)	30.4% (34)
Time between thawing and assessment (h)	3.61 ± 1.15 (1.01; 5.71)	3.52 ± 1.14 (0.09; 5.88)

For categorical variables % (n) is presented and for continuous variables mean ± SD (min; max) is presented.

Post-thaw blastocyst morphology and prediction of live birth

At the end of this study period, a subgroup of the total population, 286 blastocysts (from 220 patients), were prospectively graded for post-thaw parameters: degree of re-expansion, degree of viability and degree of cell contour. Of these, 243 blastocysts (211 patients) were transferred in single frozen–thawed blastocyst transfer cycles. The live birth rate was 46.1%. All patient and cycle characteristics were representative of the total population except more antagonist cycles were performed in this subgroup (71% compared with 62.6%) and the BMI.

Distributions of blastocyst scores for each post-thaw characteristic are summarized in Table III. The majority of blastocysts transferred had >60% re-expansion, 80% viability and 70% cell contour.

Comparable to the total population, univariate analysis of patient and cycle characteristics showed that the odds of live birth decreased for each year of female age (OR 0.93, CI 0.87–0.99, $P = 0.033$) (Table IV). In contrast, BMI was shown to be a predictor of live birth for this subgroup of patients. The odds of live birth decreased by ~17% for each increase of BMI unit (OR 0.83, CI 0.75–0.92, $P = 0.0003$). It should be noted that one-third of patient BMI values were not reported and are missing from the analysis. Furthermore, pre-freeze expansion grade, method of fertilization and pre-freeze TE grade were not shown to be significant predictors of live birth for this subgroup.

Of particular interest, the post-thaw parameters degree of re-expansion and degree of cell contour were found to be significant predictors of live birth. For each 10% increase in degree of re-expansion there was ~40% increase in odds of live birth ($P = 0.0033$). For each 10% increase in degree of cell contour, there was a 30% increase in the odds of live birth ($P = 0.029$).

The independent effect of degree of re-expansion when adjusted for significant confounders in a multivariate GEE model was an OR of 1.38 (CI 1.11–1.72, $P = 0.0042$) for each 10% increase. For cell contour adjusted for significant confounders, the odds of live birth increased by ~31% for each 10% increase (OR 1.31, CI 1.02–1.68, $P = 0.0344$).

A second stepwise logistic was performed with all significant post-thaw parameters and patient variables from the univariate analysis, except BMI (BMI was excluded from the GEE model because one-third of observations had missing values). This analysis selected degree of re-expansion and female age as the best independent predictors.

Pre-freeze and post-thaw morphology and prediction of live birth

We also performed a third stepwise logistic regression analysis to select from significant pre- and post-thaw parameters and patient variables. However, because pre-freeze morphological parameters were not found to be significant predictors of live birth for this subgroup, this analysis yielded the same results as above (see Materials and Methods).

Relationships between morphological parameters, time and day of freeze

Pearson correlations were performed between the post-thaw parameters for all blastocysts of the subgroup population, including blastocyst thawed but not transferred. The results in Table V show that all post-thaw parameters were significantly positively correlated with each other. Degree of re-expansion explained 53% of the variance in degree of viable cells and 73% of the variance in cell contour. Degree of viable cells explained 72% of the variance of the degree of cell contour.

Additionally, as shown in Table VI, all post-thaw parameters were related to pre-freeze TE grade. Cell contour was also found to correlate with pre-freeze expansion grade. However, none of these correlations explained >4% of the variation in the other variables and time did not have a significant effect on these correlations.

To determine whether time (time between warming and grading) was related to post-thaw parameter scores, Pearson correlation coefficients were calculated. A significant positive correlation was found for time and degree of re-expansion ($P = 0.034$, $r = 0.1255$), but the r^2 value was low and time only explained 2% of the variation. The other post-thaw parameters did not significantly correlate with time.

GEE models were also performed for analyzing the effect of day of cryopreservation on pre-freeze and post-thaw parameter scores. It

Table IV Post-thaw graded blastocysts: univariable GEE models for prediction of live birth.

Variable	Value	Live birth % (*n*)	Odds ratio (95% CI)	*P*-value
Female age	≤37.0	53.2% (74)		
	>37.0	36.5% (38)	0.93 (0.87–0.99)	<0.033
BMI (kg/m^2)	≤22.8	51.2% (44)		
	>22.8	36.5% (31)	0.83 (0.75–0.92)	0.0003
Indication for cause of infertility	Male	50.0% (39)	1.32 (0.70–2.47)	0.39
	Female	43.0% (34)		
	Unexplained	45.3% (39)	1.10 (0.59–2.05)	0.77
No. of earlier cycles	≤0.0	47.9% (68)		
	>0.0	43.6% (44)	0.97 (0.76–1.25)	0.83
FSH total dose (div by 500 mg)	≤3.3	51.2% (63)		
	>3.3	40.8% (49)	0.89 (0.76–1.06)	0.18
Agonist/antagonist	Antagonist	48.6% (84)		
	Agonist	40.0% (28)	0.70 (0.40–1.22)	0.21
Follicle no.	≤14.0	52.0% (65)		
	>14.0	39.8% (47)	0.98 (0.95–1.02)	0.32
Aspirated oocyte no.	≤11.0	48.6% (70)		
	>11.0	42.4% (42)	0.98 (0.93–1.03)	0.36
Method	Standard IVF	44.0% (59)		
	Microinjection	48.6% (53)	1.20 (0.72–1.99)	0.49
No. of good-quality embryo	≤3.0	43.8% (64)		
	>3.0	49.5% (48)	1.07 (0.91–1.26)	0.43
Day of freeze	5	50.3% (73)		
	6	39.8 (39)	0.65 (0.38–1.11)	0.11
Expansion	3	36.5% (31)		
	4	52.7% (78)		
	5	42.9% (3)	1.43 (0.88–2.32)	0.15
Inner cell mass	A	45.2% (70)		
	B	48.3% (42)	1.13 (0.67–1.91)	0.65
Trophectoderm cells	A	53.3% (48)		
	B	42.1% (64)	0.64 (0.38–1.08)	0.092
Degree of re-expansion (%)	50	50.0% (1)		
	70	27.8% (5)		
	80	38.5% (15)		
	90	51.9% (27)		
	100	51.2% (64)	1.39 (1.12–1.72)	0.0033
Degree of viable cells (%)	60	100% (3)		
	70	41.7% (5)		
	80	33.3% (20)		
	90	49.5% (48)		
	100	50.7% (36)	1.15 (0.86–1.53)	0.34
Degree of cell contour (%)	60	33.3% (2)		
	70	38.9% (14)		
	80	41.3% (31)		
	90	44.9% (31)		
	100	59.6% (34)	1.31 (1.03–1.68)	0.029
Time between thawing and assessment (h)	≤3.7	50.8% (62)		
	>3.7	41.3% (50)	0.93 (0.75–1.16)	0.52

Live birth rate (%) is stated for patients with the variable value. The total number of patients with each variable value can be calculated by dividing 100% by the live birth rate stated (%) and then multiplying it by (*n*).
For descriptive purposes, continuous variables have been dichotomized to less than or equal to the median value and greater than the median value, but original continuous variables were analyzed in the GEE model.
FSH total dose has been divided by 500 to facilitate interpretation of the OR (95% CI).
GEE models have been used as they allow adjustment of within-individual correlation.
For indication for cause of infertility, ORs for male versus female and unexplained versus female are presented.

Table V Pearson correlations between post-thaw parameters.

	Re-expansion	Viability	Cell contour
Degree of re-expansion (%)	1.0	0.726 (<0.0001)	0.852 (<0.0001)
Degree of viable cells (%)	0.726 (<0.0001)	1.0	0.848 (<0.0001)
Degree of cell contour (%)	0.852 (<0.0001)	0.848(<0.0001)	1.0

Pearson coefficient *r* value is shown and the *P*-value is stated in brackets ().

Table VI Pearson correlations between post-thaw and pre-freeze morphology.

	Expansion grade	ICM grade	TE grade
Degree of re-expansion (%)	−0.087 (NS)	0.002 (NS)	−0.148 (0.013)
Degree of viable cells (%)	−0.079 (NS)	−0.083 (NS)	−0.185 (0.002)
Degree of cell contour (%)	−0.142 (0.016)	−0.044 (NS)	−0.206 (0.0005)

Pearson coefficient *r* value is shown and the *P*-value is stated in brackets (). NS means non-significant.

was shown that Day 6 blastocysts had significantly higher grades of expansion ($P < 0.0001$), but significantly lower grades of ICM ($P < 0.0001$) and TE ($P = 0.0530$) than blastocysts vitrified on Day 5.

Day of cryopreservation, from Day 5 to Day 6, was significantly negatively related to all the three post-thaw parameters: degree of re-expansion ($P = 0.030$), degree of viable cells ($P = 0.0003$) and degree of cell contour ($P = 0.0004$). Linear regression analysis showed that day of freeze explained only 2% of variation in degree of re-expansion, 8% of the variation in degree of viable cells and 7% of the variation in degree of cell contour.

Discussion

Most significantly, this retrospective study has shown that pre-freeze blastocoele expansion and TE cells are the most significant morphological predictors of live birth after frozen–thawed single blastocyst transfer. Furthermore, we have shown that degree of re-expansion was the most significant post-thaw morphological predictor of live birth.

A review of the literature shows many studies contradictory to our finding that blastocysts with higher expansion grades have higher chances of live birth. Many studies have instead found that blastocysts at earlier stages of development and with lower expansion grades have better survival, implantation and live birth rates (Cho *et al.*, 2002; Mukaida *et al.*, 2003a; Van Den Abbeel *et al.*, 2005; Zech *et al.*, 2005; Ebner *et al.*, 2009). However, before comparing results, it is important to consider the method of vitrification used. The above-mentioned studies did not use artificial shrinkage of the blastocoelic cavity for higher grades of blastocysts. For these studies the blastocoelic fluid was considered an obstacle to the dehydration and permeation of the cryoprotectants and had a negative effect on the survival and pregnancy rates. However, once artificial shrinkage of the blastocoele is performed, blastocysts of higher expansion grades have been shown to have equal or even higher survival, implantation and live birth rates than blastocysts at earlier stages of development, in support of our study (Vanderzwalmen *et al.*, 2002; Son *et al.*, 2003; Mukaida *et al.*, 2006; Wikland *et al.*, 2010). The significance of pre-freeze expansion grade for live birth may be associated with more developed blastocysts having an advantageous higher number of smaller blastomeres. It has been shown that smaller blastomeres, with greater surface to volume ratio, are less sensitive to osmotic stress and injury. This is because cryoprotectants permeate faster in and out of smaller cells (Tachikawa *et al.*, 1993). As such, fully expanded blastocysts with smaller blastomeres would most likely have better tolerance to vitrification and the toxicity of high cryoprotectant concentrations. Because 70% of blastomeres are part of the TE, the same reasoning could also explain our finding and that shown by Honnma *et al.*, that pre-freeze TE is one of the most significant predictors of live birth outcomes (Honnma *et al.*, 2012).

Of particular interest, the predictive strength of expansion grade and TE in relation to live birth after frozen–thawed cycles has also been found for blastocysts transferred in fresh IVF cycles (Zaninovic *et al.*, 2001; Racowsky *et al.*, 2003; Ahlstrom *et al.*, 2011). These comparisons suggest that the vitrification method we used had little effect on the viability of blastocysts and the predictive value of important morphological parameters. As discussed in our previous study, TE has many important functions during the early stages of implantation, immediate to embryo transfer. These functions include hCG-mediated signaling, hatching from the zona pellucida, adhesion and invasion of the endometrium and communication with the maternal immune system (Licht *et al.*, 2001a,b; Jones *et al.*, 2008; Tsampalas *et al.*, 2010; Alfarawati *et al.*, 2011; Parks *et al.*, 2011). All of these functions are important in establishment of a successful pregnancy and prevention of pregnancy loss and may explain our findings that TE appearance provides more predictive information than ICM morphology at these earlier stages of development.

There are two findings in this study that are hard to explain. The first was the significance of method of fertilization and prediction of live birth. These results suggest that blastocysts developing from oocytes fertilized by ICSI are preferable for transfer in order to maximize the chance of a live birth. We have no reasonable explanation for this finding. The second finding was the importance of BMI when predicting live birth for the subgroup population, but not for the total population. We speculate that this is more likely a result of poor documentation and more apparent in the subgroup population in a shorter time period than the total population recorded over a longer time period.

Another important finding of this study was that degree of re-expansion is the most significant post-thaw morphological predictor of live birth. Re-expansion has been used by many investigators to indicate blastocyst survival after vitrification and warming (Cho *et al.*, 2002; Vanderzwalmen *et al.*, 2002, 2003; Son *et al.*, 2007). However, in most of these studies the authors waited 24 h before assessing blastocyst viability. Waiting 24 h has the disadvantage of warming more blastocysts than intended to transfer. The predictive value of re-expansion, assessed within a few hours after warming, has however been investigated for slow-freeze methods. For example, Shu *et al.* showed that fast re-expanding blastocysts (within 2–4 h) had more than double the clinical pregnancy rate when compared with non-fast re-expanded blastocysts (Shu *et al.*, 2009). This was also showed by a small study conducted by Desai and Goldfarb (2005). The majority of blastocysts warmed in our study were assessed for re-expansion for 1–5 h with a mean time of 3.5 h. We also show that time had little effect on the degree of re-expansion once the 2 h mark was reached, as blastocysts allowed greater lengths of time before assessment did not necessarily have greater degrees of expansion. This suggests that degree of re-expansion is a direct morphological response after thawing and as discussed by Ebner *et al.* a delay in this process may indicate poor cryosurvival and disturbance of water transport mechanisms (Ebner *et al.*, 2009).

Post-thaw cell contour was also shown in a multivariate analysis to significantly predict live birth indicating the importance of maintaining the integrity of the ICM and TE structures. Of similarity, Goto *et al.* showed that blastocysts allocated lower grades of ICM and TE after vitrification (i.e. down-graded scores) had significantly lower pregnancy rates (Goto *et al.*, 2011). Furthermore, degree of re-expansion was shown to positively correlate with degree of cell contour. This correlation may help when selecting blastocysts in a limited time frame as good cell contour can often be seen at lower degrees of re-expansion. A high degree of cell contour can then indicate an expected high degree of re-expansion, and for lower degrees of cell contour, it may be intuitive to warm another sibling blastocyst if available.

For obvious ethical reasons, we are not willing to transfer all embryos regardless of morphology. This in turn limits the ability to assess poorer grades of post-thaw morphology. In our study, degree of viability was not found to significantly predict live birth, most probably because all embryos transferred had >60% cell viability. We did, however, find that degree of viability was significantly related to both cell contour and degree of re-expansion, indicating the negative impact of blastomere loss on the other predictive parameters.

In our study, morphologies of blastocysts cryopreserved on Day 5 compared with Day 6 were significantly different, but encouragingly no significant difference was found in the live birth rate, 39.3 versus 38.2%. Similar findings have been reported previously (Behr *et al.*, 2002; Veeck *et al.*, 2004). In the literature, it has been much debated whether a lower rate of development affects outcomes of cryopreserved blastocysts (Shoukir *et al.*, 1998; Behr *et al.*, 2002; Desai and Goldfarb, 2005; Levens *et al.*, 2008). Even so, many clinics allow an extra day of culture for blastocyst development, aiming to increase the number of surplus embryos cryopreserved and available for patients to use in subsequent cycles. Reassuringly, our study supports this practice. Furthermore, if we compare these results with our own previous published data (Ahlstrom *et al.*, 2011), the live birth rates after fresh Day 5 blastocyst transfer (37.8%) and frozen–thawed transfer cycles (38.9%) were not significantly different. However, it is important to consider that a much higher standard of blastocyst quality (≥3BB) is required for cryopreservation compared with fresh blastocyst transfer. Therefore, a more equal comparison would be to look at the live birth rate found in another previous study performed at our clinic (Hardarson *et al.*, 2012). In this study, only good-quality blastocysts were transferred on Day 5 (≥3BB) and patients were required to have more than two GQEs available before randomization (the primary inclusion criteria), mimicking the patient and treatment characteristics required for cryopreservation on Day 5. The reported live birth rate after fresh Day 5 transfer (all ongoing pregnancies resulted in a live birth after the study was completed) for the control group was 45.6%, ~7% greater than Day 5 frozen–thawed transfers as reported here. These results suggest a difference in live birth rates between fresh and frozen–thawed Day 5 transfer of GQEs. As we do not perform transfers on Day 6, no comparisons can be made between fresh and frozen–thawed transfers performed in our clinic. However, a very recent report showed that Day 6 frozen–thawed blastocysts have a significantly higher ongoing pregnancy rate than fresh Day 6 blastocyst transfers (Shapiro *et al.*, 2012). Importantly, this study performed single-embryo transfer and compared morphologically equivalent blastocysts, unlike many other studies. This study supports previous suggestions that impaired results of Day 6 transfers are due to poor embryo-endometrium synchrony (Shapiro *et al.*, 2001, 2008; Barrenetxea *et al.*, 2005). Interestingly, it has also been demonstrated recently that frozen–thawed Day 7 blastocysts can have reasonable live birth rates when transferred to endometrium synchronized to Day 5 (Mesut *et al.*, 2012).

A limitation of this study was the small number of transferred blastocysts that were graded for post-thaw morphology limiting the amount of data available for analysis. Even so, our results do encourage the continued use of these post-thaw parameters for determination of the fate of frozen–thawed blastocysts within our clinic.

In summary, for frozen–thawed blastocyst transfer cycles blastocysts with higher pre-freeze grades of expansion and TE, irrespective of day of cryopreservation, should be given priority when thawing. Subsequently, re-expanding blastocysts, assessed within 2–4 h, with >60% viability should be transferred. For warmed blastocysts with lower rates of re-expansion, degree of cell contour can be used to determine the fate of the embryo.

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Authors' roles

All the authors, A.A, C.W., M.W. and T.H, played a role in study conception and design, took part in collection and assembly of data, carried out data analysis and interpreted the findings and drafted the manuscript. All the authors critically reviewed and approved the final version of the manuscript.

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Conflict of interest

None declared.

References

Ahlstrom 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**:3289–3296.

Alfarawati S, Fragouli E, Colls P, Stevens J, Gutierrez-Mateo C, Schoolcraft WB, Katz-Jaffe MG, Wells D. The relationship between blastocyst morphology, chromosomal abnormality, and embryo gender. *Fertil Steril* 2011;**95**:520–524.

Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology. Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. *Reprod Biomed Online* 2011;**22**:632–646.

Barrenetxea G, Lopez de Larruzea A, Ganzabal T, Jimenez R, Carbonero K, Mandiola M. Blastocyst culture after repeated failure of cleavage-stage embryo transfers: a comparison of day 5 and day 6 transfers. *Fertil Steril* 2005;**83**:49–53.

Behr B, Gebhardt J, Lyon J, Milki AA. Factors relating to a successful cryopreserved blastocyst transfer program. *Fertil Steril* 2002; **77**:697–699.

Cho HJ, Son WY, Yoon SH, Lee SW, Lim JH. An improved protocol for dilution of cryoprotectants from vitrified human blastocysts. *Hum Reprod* 2002;**17**:2419–2422.

Desai N, Goldfarb J. Examination of frozen cycles with replacement of a single thawed blastocyst. *Reprod Biomed Online* 2005;**11**:349–354.

Ebner T, Vanderzwalmen P, Shebl O, Urdl W, Moser M, Zech NH, Tews G. Morphology of vitrified/warmed day-5 embryos predicts rates of implantation, pregnancy and live birth. *Reprod Biomed Online* 2009;**19**:72–78.

Gardner DK, Schoolcraft WB. *In virto* culture of human blastocyst. In: Janson R, Mortimer D (eds). *Towards Reproductive Certainty: Infertility and Genetics Beyond 1999*. Carnforth: Parthenon Press, 1999,378–388.

Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertil Steril* 2000;**73**:1155–1158.

Goto S, Kadowaki T, Tanaka S, Hashimoto H, Kokeguchi S, Shiotani M. Prediction of pregnancy rate by blastocyst morphological score and age, based on 1,488 single frozen-thawed blastocyst transfer cycles. *Fertil Steril* 2011;**95**:948–952.

Hardarson T, Westin C, Ahlström A, Reismer E, Selleskog U, Wennerström S, Wigander A, Wikland M. Vitrification and warming human blastocyst by use of a cryoloop, using lase to artificially induce blastocyst collapse prior to vitrification. *Acta Obstet Gynecol Scand* 2007;**86**:115–128.

Hardarson T, Ahlstrom A, Rogberg L, Botros L, Hillensjo T, Westlander G, Sakkas D, Wikland M. Non-invasive metabolomic profiling of Day 2 and 5 embryo culture medium: a prospective randomized trial. *Hum Reprod* 2012;**27**:89–96.

Honnma H, Baba T, Sasaki M, Hashiba Y, Ohno H, Fukunaga T, Endo T, Saito T, Asada Y. Trophectoderm morphology significantly affects the rates of ongoing pregnancy and miscarriage in frozen-thawed single-blastocyst transfer cycle in vitro fertilization. *Fertil Steril* 2012; **98**:361–367.

Jones GM, Cram DS, Song B, Kokkali G, Pantos K, Trounson AO. Novel strategy with potential to identify developmentally competent IVF blastocysts. *Hum Reprod* 2008;**23**:1748–1759.

Lane M, Schoolcraft WB, Gardner DK. Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. *Fertil Steril* 1999;**72**:1073–1078.

Levens ED, Whitcomb BW, Hennessy S, James AN, Yauger BJ, Larsen FW. Blastocyst development rate impacts outcome in cryopreserved blastocyst transfer cycles. *Fertil Steril* 2008;**90**:2138–2143.

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* 2001a;**1**:10–19.

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* 2001b;**19**:37–47.

Liebermann J. Vitrification of human blastocysts: an update. *Reprod Biomed Online* 2009;**19**(Suppl 4):4328.

Liebermann J, Tucker MJ. Comparison of vitrification and conventional cryopreservation of day 5 and day 6 blastocysts during clinical application. *Fertil Steril* 2006;**86**:20–26.

Mesut N, Ciray HN, Mesut A, Isler A, Bahceci M. Thaw-transfers of blastocysts cryopreserved between days five to seven: a comparison of clinical outcomes. *Hum Reprod* 2012;**27**:P-148.

Mukaida T, Nakamura S, Tomiyama T, Wada S, Oka C, Kasai M, Takahashi K. Vitrification of human blastocysts using cryoloops: clinical outcome of 223 cycles. *Hum Reprod* 2003a;**18**:384–391.

Mukaida T, Takahashi K, Kasai M. Blastocyst cryopreservation: ultrarapid vitrification using cryoloop technique. *Reprod Biomed Online* 2003b; **6**:221–225.

Mukaida T, Oka C, Goto T, Takahashi K. Artificial shrinkage of blastocoeles using either a micro-needle or a laser pulse prior to the cooling steps of vitrification improves survival rate and pregnancy outcome of vitrified human blastocysts. *Hum Reprod* 2006;**21**:3246–3252.

Parks JC, McCallie BR, Janesch AM, Schoolcraft WB, Katz-Jaffe MG. Blastocyst gene expression correlates with implantation potential. *Fertil Steril* 2011;**95**:1367–1372.

Racowsky C, Combelles CM, Nureddin A, Pan Y, Finn A, Miles L, Gale S, O'Leary T, Jackson KV. Day 3 and day 5 morphological predictors of embryo viability. *Reprod Biomed Online* 2003;**6**:323–331.

Shapiro BS, Richter KS, Harris DC, Daneshmand ST. A comparison of day 5 and day 6 blastocyst transfers. *Fertil Steril* 2001;**75**:1126–1130.

Shapiro BS, Daneshmand ST, Garner FC, Aguirre M, Ross R. Contrasting patterns in in vitro fertilization pregnancy rates among fresh autologous, fresh oocyte donor, and cryopreserved cycles with the use of day 5 or day 6 blastocysts may reflect differences in embryo-endometrium synchrony. *Fertil Steril* 2008;**89**:20–26.

Shapiro BS, Daneshmand ST, Restrepo H, Garner FC, Aguirre M, Hudson C. Matched-cohort comparison of single-embryo transfers in fresh and frozen-thawed embryo transfer cycles. *Fertil Steril* 2012; **99**:389–392.

Shoukir Y, Chardonnens D, Campana A, Bischof P, Sakkas D. The rate of development and time of transfer play different roles in influencing the viability of human blastocysts. *Hum Reprod* 1998;**13**:676–681.

Shu Y, Watt J, Gebhardt J, Dasig J, Appling J, Behr B. The value of fast blastocoele re-expansion in the selection of a viable thawed blastocyst for transfer. *Fertil Steril* 2009;**91**:401–406.

Son WY, Yoon SH, Yoon HJ, Lee SM, Lim JH. Pregnancy outcome following transfer of human blastocysts vitrified on electron microscopy grids after induced collapse of the blastocoele. *Hum Reprod* 2003;**18**:137–139.

Son WY, Lee SY, Yoon SH, Lim JH. Pregnancies and deliveries after transfer of human blastocysts derived from in vitro matured oocytes in in vitro maturation cycles. *Fertil Steril* 2007;**87**:1491–1493.

Stehlik E, Stehlik J, Katayama KP, Kuwayama M, Jambor V, Brohammer R, Kato O. Vitrification demonstrates significant improvement versus slow freezing of human blastocysts. *Reprod Biomed Online* 2005;**11**:53–57.

Tachikawa S, Otoi T, Kondo S, Machida T, Kasai M. Successful vitrification of bovine blastocysts, derived by in vitro maturation and fertilization. *Mol Reprod Dev* 1993;**34**:266–271.

Takahashi K, Mukaida T, Goto T, Oka C. Perinatal outcome of blastocyst transfer with vitrification using cryoloop: a 4-year follow-up study. *Fertil Steril* 2005;**84**:88–92.

Tsampalas M, Gridelet V, Berndt S, Foidart JM, Geenen V, Perrier d'Hauterive S. Human chorionic gonadotropin: a hormone with immunological and angiogenic properties. *J Reprod Immunol* 2010;**85**:93–98.

Van den Abbeel E, Camus M, Verheyen G, Van Waesberghe L, Devroey P, Van Steirteghem A. Slow controlled-rate freezing of sequentially cultured human blastocysts: an evaluation of two freezing strategies. *Hum Reprod* 2005;**20**:2939–2945.

Vanderzwalmen P, Bertin G, Debauche C, Standaert V, van Roosendaal E, Vandervorst M, Bollen N, Zech H, Mukaida T, Takahashi K *et al*. Births after vitrification at morula and blastocyst stages: effect of artificial reduction of the blastocoelic cavity before vitrification. *Hum Reprod* 2002;**17**:744–751.

Vanderzwalmen P, Bertin G, Debauche C, Standaert V, Bollen N, van Roosendaal E, Vandervorst M, Schoysman R, Zech N. Vitrification of human blastocysts with the Hemi-Straw carrier: application of assisted hatching after thawing. *Hum Reprod* 2003;**18**:1504–1511.

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**:1418–1427.

Wikland M, Hardarson T, Hillensjo T, Westin C, Westlander G, Wood M, Wennerholm UB. Obstetric outcomes after transfer of vitrified blastocysts. *Hum Reprod* 2010;**25**:1699–1707.

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.

Zech NH, Lejeune B, Zech H, Vanderzwalmen P. Vitrification of hatching and hatched human blastocysts: effect of an opening in the zona pellucida before vitrification. *Reprod Biomed Online* 2005; **11**:355–361.