Human Reproduction, Vol.35, No.11, pp. 2478–2487, 2020
Advance Access Publication on September 18, 2020 doi:10.1093/humrep/deaa209

human reproduction

ORIGINAL ARTICLE *Embryology*

The effect of Day 3 cell number on pregnancy outcomes in vitrified-thawed single blastocyst transfer cycles

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Submitted on April 12, 2020; resubmitted on July 18, 2020; editorial decision on July 27, 2020

STUDY QUESTION: Does cell number on Day 3 have an impact on pregnancy outcomes in vitrified-thawed single blastocyst transfer cycles?

SUMMARY ANSWER: A low Day 3 cell number (≤5 cells) was independently associated with decreased live birth rate (LBR) during single blastocyst transfer cycles in young women.

WHAT IS KNOWN ALREADY: Day 3 cell number is an effective predictor of IVF success rates when transferring cleavage stage embryos. However, the association between Day 3 blastomere number and pregnancy outcomes after blastocyst transfer is still unknown.

STUDY DESIGN, SIZE, DURATION: A retrospective cohort study of 3543 patients who underwent frozen-thawed single blastocyst transfers from January 2013 to June 2018 at a tertiary-care academic medical center.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Patients were grouped into six groups according to the Day 3 cell number: ≤4 cells, 5 cells, 6 cells, 7 cells, 8 cells and >8 cells. The primary outcome measure was LBR. A logistic regression analysis was performed to explore the independent association between Day 3 blastomere number and LBR after adjustment for some potential confounders.

MAIN RESULTS AND THE ROLE OF CHANCE: In women <35 years old, the LBR varied significantly according to Day 3 cell number, with the rate of 31.2%, 34.4%, 41.9%, 45.1%, 48.1% and 48.2% for the ≤4-cell, 5-cell, 6-cell, 7-cell, 8-cell and >8-cell groups, respectively ($P < 0.001$). This significant difference was also observed in the high- and low-quality blastocyst subgroups of young women. However, for women ≥35 years old, the rate of live birth was similar between groups. Furthermore, after accounting for confounding factors, the LBR was significantly decreased in the ≤4-cell (adjusted odds ratio (aOR): 0.62, 95% CI: 0.48–0.80, $P < 0.001$) and 5-cell (aOR: 0.73, 95% CI: 0.57–0.92, $P = 0.009$) groups as compared to the 8-cell group. Likewise, the blastocysts arising from ≤4-cell (aOR: 0.73, 95% CI: 0.57–0.93, $P = 0.010$) or 5-cell (aOR: 0.77, 95% CI: 0.61–0.97, $P = 0.024$) embryos were associated with lower clinical pregnancy rate than those from 8-cell embryos. No significant differences were observed in biochemical pregnancy rate and miscarriage rate.

LIMITATIONS, REASONS FOR CAUTION: A limitation of the current study was its retrospective design. Future prospective studies are needed to confirm our findings.

WIDER IMPLICATIONS OF THE FINDINGS: Our observations suggested that a low Day 3 cell number was related to decreased LBR after blastocyst transfer in young women, which provided vital information for clinicians in selecting blastocyst during IVF treatment.

STUDY FUNDING/COMPETING INTEREST(S): This study was supported by the National Natural Science Foundation of China (NSFC) (31770989 to Y.W.; 81671520 to Q.C.) and the Shanghai Ninth People's Hospital Foundation of China (JYLJ030 to Y.W.). The authors have no conflicts of interest to declare.

TRIAL REGISTRATION NUMBER: N/A.

Key words: Day 3 cell number / live birth rate / blastocyst transfer / frozen embryo transfer / blastocyst quality

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Introduction

The primary goal in ART is to select the embryo with high development potential, so as to achieve a single healthy live birth. Extending embryo culture to the blastocyst stage is beneficial for identifying the most viable embryo and improving the synchronization of uterus and embryo (Blake *et al.*, 2007; Papanikolaou *et al.*, 2008; Glujovsky and Farquhar, 2016). Therefore, to optimize outcomes of IVF while reduce multiple pregnancies, single blastocyst transfer strategy is recommended primarily to women with a good prognosis (Penzias *et al.*, 2017).

As recommended by Gardner and Schoolcraft (1999), a morphological grading system is routinely used for selecting blastocysts for embryo transfer. Although there is a relationship between blastocyst morphology and IVF success rate (Gardner *et al.*, 2000), literature looking at chromosomal status of good-quality blastocysts still quotes overall aneuploidy rates of 39–57% (Fragouli *et al.*, 2010). Moreover, a recent study indicated that the clinical pregnancy rate of blastocysts receiving preimplantation genetic testing was significantly higher than that of blastocysts selected only by morphological quality (70.9% vs 45.8%) (Kang *et al.*, 2016). Therefore, the predictive value of conventional morphology for identifying any one developmentally competent blastocyst is still limited. Considering that many patients receive IVF treatment without genetic screening, selecting the eligible or even optimal embryos with the highest implantation potential remains a major challenge.

Day 3 blastomere number, an important indicator for early embryo progression, has been used to assess the quality of embryos during cleavage stage. In previous studies, slow cleaving embryo transfers demonstrated a low clinical potential when transferring Day 3 embryos (Check *et al.*, 2007; Racowsky *et al.*, 2011b). Furthermore, increased cell number on Day 3 was related to improved progression to the blastocyst stage and embryo morphology (Shapiro *et al.*, 2000; Luna *et al.*, 2008; Yu *et al.*, 2018). However, the effect of Day 3 cell number on the clinical outcomes after single blastocyst transfers is still unclear. If proven to be independently predictive for live birth after single blastocyst transfer, it could be a simple and low-cost tool for ranking as no additional equipment is required.

To date, only two studies showed the possible impact of Day 3 cell number on blastocyst transfer cycles (Langley *et al.*, 2001; Racowsky *et al.*, 2003), and both studies were hampered by exceedingly small sample size. Moreover, the abovementioned studies were performed exclusively in fresh IVF cycles, without ruling out possible effects of a hyperestrogenic milieu on embryo implantation (Wei *et al.*, 2019). Thus, the situation may be different in frozen embryo transfer (FET) cycles as it provided a more physiologic uterine environment for embryo implantation (Jarvela *et al.*, 2014). Additionally, given the potential relationship between Day 3 cell number and blastocyst morphology (Luna *et al.*, 2008), it is vital to detect whether Day 3 cell number is still predictive of the pregnancy outcomes in blastocysts with similar morphology.

In recent years, advances in culture media and cryopreservation techniques have resulted in the widespread use of blastocyst transfer. It is necessary to identify a set of useful, inexpensive and non-invasive criteria to guide blastocyst selection. Therefore, the purpose of the present study is to explore the impact of Day 3 cell number on pregnancy outcomes in a large cohort of patients undergoing vitrified-thawed single blastocyst transfers.

Materials and methods

Study design and patients

A retrospective study was conducted at the Department of Assisted Reproduction of the Ninth People's Hospital of Shanghai Jiao Tong University School of Medicine. The details of the ART treatment were recorded in the ART database, as required by the Technical Standard for Human-Assisted Reproduction issued by the Chinese Ministry of Health. Patients who received a frozen single blastocyst transfer during the period from January 2013 to June 2018 were involved. Exclusion criteria were as follows: aged >40 years; previous diagnosis of acquired or congenital uterine abnormality (such as a uterine malformation, intrauterine adhesion, endometrial polyps and submucosal myomas) by hysterosalpingography and three-dimensional ultrasound; core data missing in the electronic medical records. Patients with diabetes mellitus, hypertensive disorders or thyroid diseases were also excluded. Preimplantation genetic testing is not performed in this center, so none of the women included in this study used preimplantation genetic testing. Women were included in the study only once. The study protocol was approved by the Institutional Review Board of the hospital.

Embryo culture and assessment

Blastocysts were cultured in continuous single culture medium (Irvine Scientific) throughout the entire developmental stage and incubated under oil at a 37°C, 5% O_2 and 6% CO_2 environment (the balance gas was nitrogen). Embryos were recorded for cell number and morphological grade based on the Cummins's criteria (Cummins *et al.*, 1986) at 67–69 h, the standardized and recommended time post-insemination for Day 3 (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). Blastocyst stage was scored in accordance with the criteria of Gardner and Schoolcraft (1999) on Day 5 or 6. High-quality blastocysts were defined as those that meet all of the following criteria: the expansion of the blastocyst fills the embryo completely (Grade 3), the inner cell mass (ICM) is composed of several loosely grouped cells (Grade B) and the trophectoderm (TE) is composed of few cells forming a loose epithelium (Grade B). High-quality blastocysts were at least Grade 3BB, while those lower than Grade 3BB were defined as low-quality blastocysts. Poor-quality embryos (CC) were excluded in the study. Embryos with better grades were of higher priority for transfer, irrespective of the Day 3 cell number. In the study period, there was a total of four highly trained embryologists grading the embryos. All embryos were evaluated by two embryologists before determination of the embryos score to reduce the intra-variability of grading.

Endometrial preparation and vitrification

As previously shown (Zhang *et al.*, 2019), endometrial preparation for FET was performed in modified natural cycles, stimulated cycles or artificial cycles, for women with regular menstruation, irregular menstruation or a history of thin endometrium, respectively. The vitrification

and thawing procedures were described by Kuwayama et al. (2005). Briefly, embryo vitrification was carried out via a Cryotop carrier system (Kitazato Company, Japan), in conjunction with dimethylsulfoxide–ethylene glycol–sucrose as cryoprotectants. For thawing, embryos were transferred into dilution solution in a sequential manner (1 mol/l to 0.5 mol/l to 0 mol/l sucrose).

Outcome parameters and statistical methods

The main outcome of the study was live birth rate (LBR). Secondary endpoints included the rates of biochemical pregnancy, clinical pregnancy and miscarriage. Live birth was defined as delivery of a living baby at ≥24 weeks gestation. Biochemical pregnancy was defined as a pregnancy diagnosed only by the detection of hCG in serum (14 days after embryo transfer) and that does not develop into a clinical pregnancy. Clinical pregnancy was confirmed by the observation of a gestational sac on ultrasound examination on Day 35 after FET. Miscarriage was defined as an intrauterine pregnancy loss before 24th gestational week.

The characteristics of the study cohort were described using the mean with 95% CI for continuous variables and percentages with 95% CI for categorical variables. Patients were categorized into six groups based on the cell number on Day 3: ≤4 cells, 5 cells, 6 cells, 7 cells, 8 cells and >8 cells. The main pregnancy outcomes between six groups were compared with Pearson's χ^2 test or Fisher's exact test as appropriate. Furthermore, we compared each group with the 8-cell embryo group. Since a total of five comparisons were made, $P < 0.01$ (calculated as 0.05/5) was considered statistically significant in the analysis according to Bonferroni adjustment for multiple comparison. A logistic regression analysis was performed to explore the independent effect of Day 3 cell number on pregnancy outcomes after adjustment for confounding factors. The following variables were initially included as potential confounders: female age, BMI, smoking status, duration of infertility, gravidity, parity, cause of infertility, type of cycle, Day 3 morphology grade, endometrial preparation, age of frozen embryo, individual blastocyst grades (ICM, TE and expansion stage) and endometrial thickness. Depending on the results of univariate regression and stepwise regression combined with factors that were known or suspected to be associated with pregnancy outcomes, the following confounders were finally included in the multivariable model: female age at freeze, infertility duration, gravidity (0 or ≥1), parity (0 or ≥1), type of cycle, Day 3 morphology grade, age of frozen embryo (Day 5 or 6), individual blastocyst grades (ICM, TE and expansion stage) and endometrial thickness. Using the 8-cell embryo group as reference, crude and adjusted odds ratio (aOR) of biochemical pregnancy, clinical pregnancy, miscarriage and live birth were further calculated for other categories. Given that some patients had previous embryo transfer attempts, we further stratified the analysis for first-time transfers to see if the association between pregnancy outcomes and Day 3 cell number was consistent with our previous results.

To further explore the strength of the association between each parameter (ICM, TE, expansion stage and Day 3 cell number) and LBR, we performed a multivariable logistic regression using a numerical blastocyst morphology-grading system based on the criteria established by Gardner and Schoolcraft (1999). Degree of expansion was already numerically coded by an integer from 1 to 6, whereas ICM and TE grades are represented by one letter each, with A representing the highest grade. We coded these letter grades into numeric form in the simplest possible manner, using $A = 3$, $B = 2$ and $C = 1$ (Rehman et al., 2007). Regarding Day 3 cell number, we can directly use the number of cells as a continuous variable. The standardized coefficients, odds ratio (OR) and *P*-values of each parameter were calculated. All statistical analyses were performed using SPSS version 23.0 software (SPSS Inc., Chicago, USA). Two-tailed *P*-values <0.05 were considered significant.

Results

After exclusions, a total of 3543 women were included for analysis. The characteristics of the patient cohort are presented in Table I. Briefly, the mean female age and BMI was 32.6 (95% CI 32.5–32.8) years and 21.6 (95% CI 21.5–21.7) kg/m^2, respectively. More than 90% of the women were primipara, and the primary cause of infertility was tubal factor (71.9%). These patients underwent high-quality blastocyst transfer in 65.7% of cycles, with a mean endometrial thickness of 10.4 (95% CI 10.3–10.4) mm on the day of transfer. The specifics of embryo quality and day of freezing for the Day 3 cell number embryo groups are shown in Supplementary Table SI.

The association between main pregnancy outcomes and Day 3 cell number is shown in Table II. In women aged <35 years, the rate of live birth was 31.2%, 34.4%, 41.9%, 45.1%, 48.1% and 48.2% for the ≤4-cell, 5-cell, 6-cell, 7-cell, 8-cell and >8-cell groups, respectively. LBR differed significantly between groups ($P < 0.001$). Likewise, the clinical pregnancy rate varied significantly according to Day 3 cell number for young women ($P < 0.001$). It rose with the increased Day 3 blastomere number, with the clinical pregnancy rate of 41.5%, 42.6%, 49.9%, 53.1%, 56.6% and 59.0% for the ≤4-cell, 5-cell, 6-cell, 7-cell, 8-cell and >8-cell groups, respectively. However, for women aged ≥35 years, these groups were similar in the rates of clinical pregnancy and live birth ($P = 0.735$ and 0.635, respectively). Comparison among the six groups did not reveal any statistically significant differences in miscarriage rate and biochemical pregnancy rate for all women. As shown in Fig. 1, in women <30 or 30–34 years old, LBR varied significantly between groups (both $P = 0.003$). Nonetheless, this difference was not observed in women ≥35 years of age ($P = 0.635$). For a given cell number on Day 3, the incidence of live birth decreased with increasing age.

Given that morphological quality of the blastocyst is an important determinant of IVF success, and that there was a potential relationship between cell number on Day 3 and embryo morphology, it is vital to explore the effect of Day 3 cell number in both high-quality and low-quality FET cycles. As presented in Table III, in young women who transferred high-quality embryos, the incidence of clinical pregnancy and live birth still significantly differed between the study groups ($P = 0.001$ and <0.001, respectively). This difference was also found in young women who transferred low-quality embryos. Biochemical pregnancy rate and miscarriage rate were similar across all the Day 3 blastomere number categories in both high- and low-quality blastocysts for all women.

As presented in Table IV, after adjustment for some confounding variables, the ≤4-cell (aOR 0.62, 95% CI: 0.48–0.80, $P < 0.001$) and 5-cell (aOR 0.73, 95% CI: 0.57–0.92, $P = 0.009$) groups were

Table I Baseline demographics and cycle characteristics of the study cohort.

Parameter	Value
Number of women, n	3543
Maternal age (years)	32.6 (32.5, 32.8)
BMI (kg/m^2)	21.6 (21.5, 21.7)
Infertility duration (years)	3.4 (3.3, 3.5)
Maternal smoking	0.8 (0.6, 1.2)
Gravidity	
0	52.9 (51.3, 54.6)
≥1	47.1 (45.4, 48.7)
Parity	
0	91.5 (90.5, 92.4)
≥1	8.5 (7.6, 9.5)
Cause of infertility	
Tubal	71.9 (70.3, 73.3)
Ovulatory	9.7 (8.7, 10.7)
Endometriosis	9.8 (8.8, 10.8)
Male cause	20.9 (19.6, 22.3)
Type of cycle	
IVF	65.3 (63.7, 66.9)
ICSI	26.1 (24.6, 27.5)
Combined IVF/ICSI	8.7 (7.8, 9.6)
Endometrial preparation	
Modified natural cycle	24.7 (23.3, 26.2)
Stimulated cycle	39.1 (37.5, 40.7)
Hormonal replacement	36.2 (34.6, 37.8)
Blastocyst quality	
High quality	65.7 (64.1, 67.3)
Low quality	34.3 (32.7, 35.9)
Expansion	
3	1.2 (0.9, 1.6)
4	90.5 (89.5, 91.5)
5	5.8 (5.0, 6.6)
6	2.5 (2.0, 3.1)
Inner cell mass	
A	17.6 (16.4, 18.9)
B	73.2 (71.7, 74.6)
C	9.2 (8.3, 10.2)
Trophectoderm	
A	7.0 (6.2, 7.9)
B	67.9 (66.3, 69.4)
C	25.1 (23.7, 26.6)
Days of frozen embryo	
Day 5	28.4 (26.9, 29.9)
Day 6	71.6 (70.1, 73.1)
Endometrium thickness on the day of embryo transfer (mm)	10.4 (10.3, 10.4)

For category variables, rate % (95% CI) is presented and for continuous variables, mean (95% CI) is presented.

associated with lower LBR than the 8-cell group. Also, in the multivariable model, clinical pregnancy rate was significantly decreased in the ≤4-cell (aOR 0.73, 95% CI: 0.57–0.93, $P = 0.010$) and 5-cell (aOR 0.77, 95% CI: 0.61–0.97, $P = 0.024$) groups as compared with that in the reference group. The other reproductive outcomes, including the prevalence of biochemical pregnancy and miscarriage, were similar between the different cell number groups. The details of the covariate adjustment in the multiple logistic model for LBR are listed in Table V. The LBR of ICM A (aOR 2.17, 95% CI: 1.58-2.98, $P < 0.001$) and B (aOR 1.41, 95% CI: 1.07–1.85, $P = 0.004$) stage was higher than that of ICM C stage (Table V). TE B stage was associated with increased LBR as compared with TE C stage (aOR 1.29, 95% CI: 1.08–1.54, $P = 0.004$). In addition, when the analysis was restricted to first-time transfers, the association between pregnancy outcomes and Day 3 cell number did not change (Supplementary Table SII).

In multivariable regression model using the abovementioned numerical blastocyst morphology-grading system (Supplementary Table SIII), after adjustment for the other confounders, the standardized coefficients, OR, and *P*-values of the Day 3 cell number, ICM, TE and expansion were 0.086, 0.103, 0.060 and −0.001; 1.110, 1.448, 1.227 and 0.995; and <0.001, <0.001, 0.003 and 0.955, respectively.

Discussion

It is a critical issue to select the most viable embryos for transfer during IVF treatment. Although morphological assessment has been the primary and most commonly used approach, the efficiency of morphological quality is low for IVF treatment. Therefore, there is certainly a need for improvements in the effectiveness of the method. Our findings showed that a low Day 3 cell number (≤5 cells) was independently associated with decreased rate of live birth after single blastocyst transfers. Moreover, whether it was a high-quality embryo or a low-quality embryo, the cell number on Day 3 was an important parameter that could effectively predict the LBR in young women, which provided vital information for blastocyst selection in IVF treatment.

To date, only two studies have been interested in the potential association between Day 3 cell number and outcomes of blastocyst transfer cycles. Racowsky *et al.* (2003) reported that blastocysts resulting from lower (<7 cells) cleavage stages were not related to reduced blastocyst viability. Nonetheless, a total of 194 blastocysts were involved, of which only 14 blastocysts resulted from <7-cell embryos. The ongoing pregnancy rate of blastocysts in the <7-cell group was lower than that of blastocysts in the 7- to 8-cell group (42.9% vs 55.2%). However, most likely due to the small sample size, this difference did not reach statistical significance. Langley *et al.* (2001) reported that low Day 3 cell number was related to decreased implantation rate. However, the authors acknowledged that the study population in this study was insufficient, with only 23 women in 3–4 cells group and 34 women in 5–6 cells group, making the result difficult for robust conclusions.

Notably, the incidence of live birth, miscarriage and biochemical pregnancy was not reported in the aforementioned studies. Moreover, the grouping method applied in these literatures was too simple to

Table II Pregnancy outcomes grouped by Day 3 cell number.

	≤4 cells	5 cells	6 cells	7 cells	8 cells	>8 cells	*P*-value
Female age < 35	n = 388	n = 430	n = 599	n = 486	n = 387	n = 83	
Biochemical pregnancy rate	5.2 (3.2, 7.8)	5.3 (3.4, 7.9)	4.5 (3.0, 6.5)	6.0 (4.0, 8.5)	4.1 (2.4, 6.6)	7.2 (2.7, 15.1)	0.700
Miscarriage rate	10.3 (7.5, 13.8)	8.1 (5.7, 11.1)	8.0 (6.0, 10.5)	8.0 (5.8, 10.8)	8.5 (5.9, 11.8)	10.8 (5.1, 19.6)	0.767
Clinical pregnancy rate	41.5 (36.5, 46.6)*	42.6 (37.8, 47.4)*	49.9 (45.8, 54.0)	53.1 (48.5, 57.6)	56.6 (51.5, 61.6)	59.0 (47.7, 69.7)	<0.001
Live birth rate	31.2 (26.6, 36.1)*	34.4 (29.9, 39.1)*	41.9 (37.9, 46.0)	45.1 (40.6, 49.6)	48.1 (43.1, 53.2)	48.2 (37.1, 59.4)	<0.001
Female age ≥ 35	n = 192	n = 253	n = 287	n = 209	n = 194	n = 35	
Biochemical pregnancy rate	4.2 (1.8, 8.0)	5.9 (3.4, 9.6)	4.2 (2.2, 7.2)	5.7 (3.0, 9.8)	4.1 (1.8, 8)	0.0 (–)	0.704
Miscarriage rate	12.0 (7.7, 17.4)	9.9 (6.5, 14.2)	10.1 (6.9, 14.2)	11.5 (7.5, 16.6)	8.2 (4.8, 13)	8.6 (1.8, 23.1)	0.854
Clinical pregnancy rate	40.1 (33.1, 47.4)	41.1 (35.0, 47.4)	39.7 (34.0, 45.6)	45.5 (38.6, 52.5)	42.8 (35.7, 50.1)	34.3 (19.1, 52.2)	0.735
Live birth rate	28.1 (21.9, 35.1)	31.2 (25.6, 37.3)	29.6 (24.4, 35.3)	34.0 (27.6, 40.8)	34.5 (27.9, 41.7)	25.7 (12.5, 43.3)	0.635

Data are presented as rate % (95% CI).
*$P < 0.01$ (which was considered statistically significant in the analysis according to Bonferroni adjustment for multiple comparison) using the 8-cell embryo group as reference.

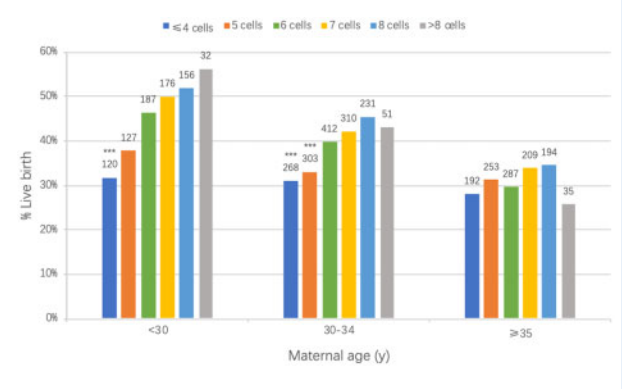

Figure 1. Live birth rate according to Day 3 cell number and maternal age. ***$P < 0.01$ (which was considered statistically significant in the analysis according to Bonferroni adjustment for multiple comparison) using the 8-cell embryo group as reference. According to Pearson χ^2 test, in women <30, 30–34 and ≥35 years old, the *P*-values for comparisons between the six groups were 0.003, 0.003 and 0.635, respectively. The n-values are shown above the bars.

show the trend of pregnancy outcome in different cell number groups. In addition, previous studies were conducted in fresh embryo transfer cycles and therefore, the possible harm of supraphysiologic hormonal milieu during controlled ovarian stimulation could not be ruled out.

The present study, aiming to improve on the flaws of previous studies, looked at the exact role of Day 3 cell number in blastocyst viability. Our results, based on 3543 vitrified-thawed single blastocyst transfer cycles, clearly demonstrated that a low Day 3 cell number itself had an impact on IVF outcomes.

Recently, time-lapse imaging technology, which can capture frequent images without disturbing culture conditions, has been applied to human embryos. It can assess morphokinetic variables from fertilization to cleavage and blastocyst stages and further enhance IVF outcomes (Conaghan *et al.*, 2013; Herrero *et al.*, 2013; Adamson *et al.*, 2016; Motato *et al.*, 2016). Although effectiveness of such morphokinetic markers for predicting blastocyst development potential is still controversial, some studies have reported that the timing of early events during embryonic development differed between non-implanted and implanted blastocysts (Chamayou *et al.*, 2013; Desai *et al.*, 2014). Furthermore, a systematic review on this topic demonstrated that the times to 2-cell, 3-cell, 4-cell, 5-cell and 8-cell stages were longer in non-implanted blastocysts (Kaser and Racowsky, 2014). These observations were to some extent in line with the results of our current study using conventional assessments, indicating that Day 3 blastomere number was predictive of IVF success. If time-lapse imaging technology was performed, the additional Day 3 check outside the incubator would not have to be performed, and instead, time-lapse algorithms could be used in place of this. However, for the embryos not using time-lapse, it is necessary to perform an additional morphology check on Day 3 of development, considering that Day 3 cell number is an effective indicator that could be independently predictive of LBR in blastocyst transfer cycles.

The reason why a low number of cells on Day 3 leads to a decreased LBR remains unclear. It is speculated that a difference in aneuploidy rate may play a role. Indeed, a recent retrospective study including 4028 embryos reported that embryos with 4–5 cells (OR: 0.18, 95% CI: 0.10–0.31), 6 cells (OR: 0.29, 95% CI: 0.18–0.44) and 7 cells (OR: 0.44, 95% CI: 0.32–0.60) on Day 3 had significantly reduced likelihood of becoming euploid blastocysts as compared with 8-cell Day 3 embryos (Pons *et al.*, 2019). As a consequence, the chromosomal abnormalities may result in implantation failure and abortion in IVF cycles. Of note, a positive association was found between female age and the occurrence of aneuploidy (Franasiak *et al.*, 2014; Minasi *et al.*, 2016). Although there is no clear evidence for any simple explanation of the impact of female age on aneuploidy rate, it has been suggested that environmental and intrinsic factors may influence the meiotic segregation of chromosomes according to age (Pellestor *et al.*, 2003). Therefore, the inherently high aneuploidy rate of older women may attenuate the effect of cell number on embryo chromosomes, which may be the reason why Day 3 cell number had less effect on older women in the present study.

Table III Association between pregnancy outcomes and Day 3 cell number in high-quality and low-quality blastocyst transfer.

		≤4 cells	5 cells	6 cells	7 cells	8 cells	>8 cells	*P*-value
Age <35	**High-quality blastocysts**	n = 241	n = 265	n = 389	n = 351	n = 285	n = 54	
	Biochemical pregnancy rate	5.8 (3.2, 9.6)	6.0 (3.5, 9.6)	3.9 (2.2, 6.3)	5.7 (3.5, 8.7)	4.6 (2.5, 7.7)	1.9 (0.0, 9.9)	0.606
	Miscarriage rate	10.8 (7.2, 15.4)	8.7 (5.6, 12.7)	6.9 (4.6, 9.9)	8.3 (5.6, 11.7)	9.5 (6.3, 13.5)	11.1 (4.2, 22.6)	0.619
	Clinical pregnancy rate	43.2 (36.8, 49.7)*	49.4 (43.3, 55.6)	53.5 (48.4, 58.5)	55.6 (50.2, 60.8)	57.5 (51.6, 63.4)	68.5 (54.4, 80.5)	0.001
	Live birth rate	32.4 (26.5, 38.7)*	40.8 (34.8, 46.9)	46.5 (41.5, 51.6)	47.3 (42.0, 52.7)	48.1 (42.1, 54.0)	57.4 (43.2, 70.8)	<0.001
	Low-quality blastocysts	n = 147	n = 165	n = 210	n = 135	n = 102	n = 29	
	Biochemical pregnancy rate	4.1 (1.5, 8.7)	4.2 (1.7, 8.5)	5.7 (3.0, 9.8)	6.7 (3.1, 12.3)	2.9 (0.6, 8.4)	17.2 (5.8, 35.8)	0.058
	Miscarriage rate	9.5 (5.3, 15.5)	7.3 (3.8, 12.4)	10.0 (6.3, 14.9)	7.4 (3.6, 13.2)	5.9 (2.2, 12.4)	10.3 (2.2, 27.4)	0.795
	Clinical pregnancy rate	38.8 (30.9, 47.2)	31.5 (24.5, 39.2)*	43.3 (36.5, 50.3)	46.7 (38.0, 55.4)	53.9 (43.8, 63.8)	41.4 (23.5, 61.1)	0.009
	Live birth rate	29.3 (22.0, 37.3)*	24.2 (17.9, 31.5)*	33.3 (27.0, 40.1)	39.3 (31.0, 48.0)	48.0 (38.0, 58.2)	31.0 (15.3, 50.8)	0.002
Age ≥35	**High-quality blastocysts**	n = 119	n = 146	n = 183	n = 136	n = 135	n = 24	
	Biochemical pregnancy rate	5.0 (1.9, 10.7)	7.5 (3.8, 13.1)	3.3 (1.2, 7.0)	7.4 (3.6, 13.1)	3.7 (1.2, 8.4)	0.0 (–)	0.895
	Miscarriage rate	11.8 (6.6, 19.0)	7.5 (3.8, 13.1)	10.9 (6.8, 16.4)	11.0 (6.3, 17.5)	7.4 (3.6, 13.2)	12.5 (2.7, 32.4)	0.711
	Clinical pregnancy rate	44.5 (35.4, 53.9)	42.5 (34.3, 50.9)	43.2 (35.9, 50.7)	51.5 (42.8, 60.1)	43.0 (34.5, 51.8)	29.2 (12.6, 51.1)	0.363
	Live birth rate	32.8 (24.4, 42.0)	34.9 (27.2, 43.3)	32.2 (25.5, 39.5)	40.4 (32.1, 49.2)	35.6 (27.5, 44.2)	16.7 (4.7, 37.4)	0.291
	Low-quality blastocysts	n = 73	n = 107	n = 104	n = 73	n = 59	n = 11	
	Biochemical pregnancy rate	2.7 (0.3, 9.5)	3.7 (1.0, 9.3)	5.8 (2.1, 12.1)	2.7 (0.3, 9.5)	5.1 (1.1, 14.1)	0.0 (–)	0.844
	Miscarriage rate	12.3 (5.8, 22.1)	13.1 (7.3, 21.0)	8.7 (4.0, 15.8)	12.3 (5.8, 22.1)	10.2 (3.8, 20.8)	0.0 (–)	0.743
	Clinical pregnancy rate	32.9 (22.3, 44.9)	39.3 (30.0, 49.2)	33.7 (24.7, 43.6)	34.2 (23.5, 46.3)	42.4 (29.6, 55.9)	45.5 (16.7, 76.6)	0.770
	Live birth rate	20.5 (12.0, 31.6)	26.2 (18.1, 35.6)	25.0 (17.0, 34.4)	21.9 (13.1, 33.1)	32.2 (20.6, 45.6)	45.5 (16.7, 76.6)	0.395

Data are presented as rate % (95% CI).
*$P < 0.01$ (which was considered statistically significant in the analysis according to Bonferroni adjustment for multiple comparison) using the 8-cell embryo group as reference.

Table IV Crude and adjusted odds ratios (ORs) for main pregnancy outcomes after single blastocyst transfers.

	≤4 cells	5 cells	6 cells	7 cells	8 cells	>8 cells
Biochemical pregnancy rate						
Crude OR (95% CI)	1.18 (0.67, 2.06)	1.37 (0.81, 2.31)	1.07 (0.64, 1.80)	1.46 (0.87, 2.44)	Reference	1.24 (0.50, 3.11)
Adjusted OR (95% CI)	1.17 (0.67, 2.07)	1.34 (0.78, 2.28)	1.05 (0.62, 1.80)	1.46 (0.87, 2.45)	Reference	1.23 (0.49, 3.10)
Clinical pregnancy rate						
Crude OR (95% CI)	**0.64 (0.51, 0.81)**	**0.67 (0.54, 0.84)**	**0.81 (0.65, 1.00)**	0.95 (0.77, 1.19)	Reference	0.99 (0.67, 1.47)
Adjusted OR (95% CI)	**0.73 (0.57, 0.93)**	**0.77 (0.61, 0.97)**	0.87 (0.70, 1.09)	0.95 (0.76, 1.20)	Reference	0.93 (0.62, 1.39)
Miscarriage rate						
Crude OR (95% CI)	1.32 (0.89, 1.96)	1.05 (0.71, 1.55)	1.03 (0.71, 1.50)	1.08 (0.73, 1.60)	Reference	1.23 (0.63, 2.39)
Adjusted OR (95% CI)	1.43 (0.95, 2.15)	1.12 (0.75, 1.69)	1.10 (0.75, 1.62)	1.11 (0.75, 1.65)	Reference	1.22 (0.62, 2.38)
Live birth rate						
Crude OR (95% CI)	**0.56 (0.44, 0.71)**	**0.65 (0.51, 0.81)**	**0.79 (0.64, 0.98)**	0.93 (0.74, 1.16)	Reference	0.92 (0.62, 1.38)
Adjusted OR (95% CI)	**0.62 (0.48, 0.80)**	**0.73 (0.57, 0.92)**	0.84 (0.67, 1.05)	0.92 (0.73, 1.16)	Reference	0.86 (0.57, 1.30)

Bold indicates significant *P*-values which are <0.05.
Analyses were adjusted for female age, infertility duration, gravidity, parity, type of cycle, Day 3 morphology grade, age of frozen embryo, individual blastocyst grades (inner cell mass, trophectoderm and expansion stage) and endometrial thickness.

We performed a comparison of FET cycles according to embryo morphological quality. For women aged <35 years, low cell number on Day 3 is significantly associated with decreased LBR for both high-grade and low-grade blastocyst transfer. Therefore, when selecting blastocysts, Day 3 blastomere number may be considered along with conventional morphological quality in young women. This finding may be particularly useful in elective single embryo transfer, since a high-quality blastocyst arising from an 8-cell Day 3 embryo may be more likely to be implanted. Moreover, this guidance in embryo selection does not necessitate additional equipment and is low cost. When

Table V Results of multiple regression analysis for live birth rates.

	OR	95% CI	*P*-value
Day 3 cell number			
<4 cells	0.62	0.48, 0.80	<0.001
5 cells	0.73	0.57, 0.92	0.009
6 cells	0.84	0.67, 1.05	0.126
7 cells	0.92	0.73, 1.16	0.469
8 cells	Reference		
>8 cells	0.86	0.57, 1.30	0.487
Maternal age	0.95	0.93, 0.97	<0.001
Infertility duration	0.98	0.96, 1.01	0.156
Gravidity	1.06	0.91, 1.24	0.429
Parity	0.88	0.67, 1.16	0.368
Type of cycle			
IVF	Reference		
ICSI	0.92	0.78, 1.09	0.354
Combined IVF/ICSI	1.15	0.89, 1.48	0.282
Day 3 morphology grade			
I–II	Reference		
III–IV	1.07	0.86, 1.32	0.556
Expansion			
3	Reference		
4	2.15	1.04, 4.43	0.039
5	2.08	0.95, 4.55	0.066
6	1.71	0.73, 4.03	0.221
Inner cell mass			
A	2.17	1.58, 2.98	<0.001
B	1.41	1.07, 1.85	0.004
C	Reference		
Trophectoderm			
A	1.32	0.95, 1.84	0.099
B	1.29	1.08, 1.54	0.004
C	Reference		
Age of frozen embryo			
Day 5	Reference		
Day 6	0.73	0.62, 0.86	<0.001
Endometrium thickness	1.05	1.02, 1.08	0.003

preimplantation genetic screening is not performed, Day 3 cell number, along with blastocyst morphological grade, may be beneficial to improve viable embryo selection at the time of transfer.

The developmental competence of fast-cleaving embryos is still a controversial issue. Kroener *et al.* (2015) observed that a number of Day 3 blastomeres >9 was associated with significantly increased aneuploidy rates. The ESHRE-Alpha consensus (Balaban *et al.*, 2011) stated that embryos that cleave faster than the optimal rate (8 cells) have a reduced implantation potential. Similarly, the Society for Assisted Reproductive Technology Clinic Outcome Reporting System (Racowsky *et al.*, 2011a) showed a positive association between the LBR and the increasing blastomere number up to 8 and a reduced rate in embryos with more than 8 cells. However, a more recent study showed increased LBR with increased cell number (Kong *et al.*, 2016). In the current study, the clinical pregnancy and LBRs were similar between >8-cell and 8-cell groups. Considering that the population of the >8-cell group was relatively small, future larger studies are needed to confirm this finding.

It is of vital importance to evaluate each parameter's (ICM, TE, stage and Day 3 cell number) contribution to the reproductive outcomes in IVF. According to the result of multivariable regression analysis, cell number on Day 3 is independently predictive of live birth in the presence of the other confounders, and therefore it should be considered along with conventional morphological parameters (ICM, TE and expansion stage) when selecting blastocysts. Previous studies have explored the individual effect of each variable on IVF outcomes. Some investigators have shown that expansion stage is an effective predictor of implantation (Yoon *et al.*, 2001; Shapiro *et al.*, 2008; Zhao *et al.*, 2019). However, in the current study, the expansion stage only had a slight effect on LBR, which may be due to the fact that most blastocysts we analyzed were in expansion stage 4 (90.5% (3207/3543)). Others have observed a strong association between grade of ICM and IVF success rate (Balaban *et al.*, 2000; Richter *et al.*, 2001), which is consistent with our results. Noteworthy is that TE stage has been reported to be positively associated with implantation, and its predictive strength exceeded that of ICM for selecting the best blastocyst (Zaninovic *et al.*, 2001; Ahlstrom *et al.*, 2011). However, others declare that no relationship between TE stage and pregnancy outcomes was observed (Richter *et al.*, 2001; Subira *et al.*, 2016). This controversy will persist until conclusive evidence is provided by adequately powered randomized controlled trials. Additionally, in the present study, no association between Day 3 morphology grade and LBR was observed. This result was in line with previous publications reporting the association between Day 3 morphology grade and IVF success after blastocyst transfers (Racowsky *et al.*, 2003; Herbemont *et al.*, 2017).

Previous studies showed that the LBR of low-quality blastocysts was in the range of 11.4–34.1% (Oron *et al.*, 2014; Zhu *et al.*, 2014; Bouillon *et al.*, 2017; Dobson *et al.*, 2018). In the present study, low-quality blastocysts maintained a reasonable LBR, with a total LBR of 30.7% (373/1215). This result suggests that low-quality blastocyst should not be given up during IVF treatment (Morbeck, 2017), as they maintained a reasonably high chance of live birth. Of note, all women in this study received FETs, which provided a more physiological uterine environment for embryo implantation and early fetal development. Additionally, blastocysts grade CC were excluded from the analysis, which may also lead to better IVF outcomes. Additionally, for embryos that were 8 cells on Day 3, there was little difference in the LBR between high- and low-quality blastocysts (44.0% (185/420) and 42.2% (68/161), respectively). This result suggested that the blastocyst grade (high or low) had little impact on LBR of the 8-cell group, which may be because the euploid rate of this group is significantly higher than that of the other groups (Pons *et al.*, 2019), although we primarily confirm this finding in the younger subgroup.

The main strength of our study was the large sample size; to date, this is the largest study evaluating the impact of Day 3 cell number on pregnancy outcomes after blastocyst transfers. Several relevant confounders that might otherwise have biased the findings were adjusted in the present study. Moreover, all embryos were evaluated by two

embryologists before determination of the embryo's score to reduce the intra-variability of grading and improve data quality. Additionally, Day 3 cell number was divided into more details and provide an insight into the trends of LBR after single blastocyst transfers over a different number of cells on Day 3. A limitation of the current study was its retrospective nature. There may be subtle differences in developmental timings, which may potentially affect the results. In this aspect, we included a number of confounders in the multivariable model to make the research more rigorous. Furthermore, we restricted the analysis to FET cycles, ruling out possible effects of a hyperestrogenic milieu on the embryo implantation (Wei *et al.*, 2019).

In summary, the current large single-center retrospective study showed that a low Day 3 cell number was related to reduced LBR after single blastocyst transfers in young women regardless of embryo morphology. Thus, Day 3 cell number may be used along with blastocyst morphological grade to improve embryo selection. Further studies are needed to confirm these findings.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Acknowledgements

We gratefully acknowledge all the staff of the Department of Assisted Reproduction in Shanghai Ninth People's Hospital for their support and cooperation.

Authors' roles

Y.W. supervised the entire study, including the procedures, conception, design and completion. Q.C. were responsible for the collection of data. J.Z. and Y.K. contributed to the data analysis. J.W. contributed the data analysis and drafted the article. Y.W. participated in the interpretation of the study data and in revisions to the article.

Funding

This study was supported by the National Natural Science Foundation of China (NSFC) (31770989 to Y.W.; 81671520 to Q.C.) and the Shanghai Ninth People's Hospital Foundation of China (JYLJ030 to Y.W.).

Conflict of interest

None of the authors have any conflicts of interest to declare.

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