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ASSISTED REPRODUCTION TECHNOLOGIES

Cell number considerations for blastocyst transfer in younger patients

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Abstract

Objective To investigate the role of the cell number at day 3 in blastocyst selection.

Design Observational, retrospective, single-center clinical study.

Patient(s) In part 1, 1211 single vitrified-warmed blastocyst transfer (SVBT) cycles were identified and reviewed. All the cycles were conventional in vitro fertilization (IVF) cycles and the first embryo transfer cycles. Most of patients had a risk of ovarian hyperstimulation syndrome and were young. In part 2, 864 IVF-derived blastocysts from 292 infertile couples underwent trophectoderm (TE) biopsy for preimplantation genetic testing for aneuploidies (PGT-A).

Intervention(s) No patient intervention.

Main outcome measure(s) The first part was an analysis of the correlation between the cell number at day 3 and live birth rate (LBR) after SVBT, and the second part was an analysis of the correlation between the cell number at day 3 and euploid rate (ER) of blastocysts.

Result(s) In part 1, after correcting for the effects of other confounders, the cell number at day 3 had no significant effect on the LBR (OR 1.001, 95% CI 0.938–1.068). In part 2, after correcting for the effects of other confounders, the cell number at day 3 had no significant effect on the ER (OR 0.960, 95% CI 0.866–1.063).

Conclusion(s) When the vitrified-warmed blastocysts obtained by conventional IVF are transferred into young patients, the cell number at day 3 is not a strong predictor of the LBR. In addition, the cell number at day 3 is not a strong predictor of ER of IVF-derived blastocysts too.

Keywords Blastocyst selection · Cell number · Live birth rate · Euploid rate

Introduction

With the successful introduction of blastocyst culture and embryo vitrification, a single vitrified-warmed blastocyst transfer (SVBT) has been increasingly advocated. SVBT can reduce the risks of multiple gestation pregnancies along with the risks of associated maternal and foetal complications [1], but SVBT has a lower pregnancy rate than multiple-embryo transfer. Therefore, to maintain a high pregnancy rate, selecting the best blastocyst for transfer is very important.

The most commonly used method for blastocyst selection is blastocyst morphology assessment. The major focus of the assessment is three parameters: expansion and hatching stage, inner cell mass (ICM) grade, and trophectoderm (TE) grade. It is generally accepted that TE development is a reflection of the ability of the embryo to attach to and implant in the endometrium, while ICM development is clearly crucial for the development of the foetus itself [2]. Therefore, TE is an important parameter for predicting implantation [3]. The transfer of a blastocyst with a better ICM grade may reduce the risk of early pregnancy loss [4]. A recent randomized controlled trial found that elective frozen single blastocyst transfer improves the singleton live birth rate compared with fresh single blastocyst transfer [5]. In frozen-thawed single blastocyst transfer cycles, the blastocoel expansion degree is selected as the best parameter for predicting a clinically successful pregnancy [6, 7].

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In practice, most people assess the cleavage-stage embryo morphology in combination with the three blastocyst morphological parameters to select the best blastocyst. Six parameters—cell number, fragmentation (%), symmetry, multi-nucleation, vacuoles, and zona pellucida—are used for assessing the cleavage-stage embryo morphology according to the Istanbul consensus [8]. The occurrence of cellular division is the single most important indicator of embryo viability [8], so cell number is an important parameter. Embryos with seven to eight cells at day 3 are scored as grade A. The cell number can intuitively reflect the cleavage rate. Previous studies revealed that an embryo cleavage rate that is too slow or too fast has a negative impact on the implantation potential of day-3 embryos [8, 9]. And a correlation between the cell number of day-3 embryos and their chromosomal constitution has also been reported [8, 10].

Therefore, it seems that both the cell number at day 3 and blastocyst morphology are the important evaluation parameters for embryo selection. However, although the cell number at day 3 is considered as an important parameter for cleavage-stage embryo selection, is it still an important parameter for blastocyst selection? And if it is in conflict with the blastocyst morphology in the blastocyst selection, how shall we choose: a blastocyst with better morphology or with the optimal cell number at day 3? Both of these questions are unclear. Therefore, the purpose of our study is to determine the correlation between the cell number at day 3 and either the live birth rate (LBR) after SVBT or euploid rate (ER) of blastocysts. It will help us to know the role of the cell number at day 3 in the blastocyst selection.

Materials and methods

Study design

A retrospective study was performed. This study was divided into two parts. The first part was an analysis of the correlation between the cell number at day 3 and LBR after SVBT, and the second part was an analysis of the correlation between the cell number at day 3 and ER of blastocysts.

Patients in the first part of the study

All patients were aged 20–44 years and underwent stimulation according to a GnRH agonist suppression protocol (short or long) at our hospital from January 2015 to February 2017. Each patient underwent a SVBT cycle. The SVBT cycle inclusion criteria are as follows: (1) It was the first embryo transfer cycle. (2) It was a SVBT with her own blastocyst. (3) It was a conventional IVF cycle. The main cycle exclusion criteria are as follows: (1) cycles involving frozen-thawed oocytes; (2) cycles involving gamete or embryo donation; (3) cycles involving fresh embryo transfer; (4) cycles involving preimplantation genetic testing (PGT); (5) intracytoplasmic sperm injection (ICSI) cycles. We excluded the ICSI cycles because cleavage rate may be different between embryos originating from ICSI and those originating from conventional IVF. If we included the ICSI cycles, the cell number at day 3 might not be homogenous across all the cohorts of embryos included in the study.

These patients were divided into the live birth group and no live birth group, and there were no significant differences in the duration of infertility, type of infertility, cause of infertility, or BMI between the two groups.

Embryo culture and score

Embryos were cultured in sequential medium and covered with sterile mineral oil at 37 °C under 6% CO2, 5% O2, and 89% N2 for a maximum of 7 days (usually 6 days). Zygotes (16–18 h postinsemination) that had two pronuclei (2PN) and two polar bodies were considered successfully fertilized. However, zygotes with no pronucleus (0PN) or one pronucleus (1PN) were maintained in culture. This was because some of the 0 and 1 PN embryos are diploid and contain both the maternal and paternal genomes [11]. These embryos are successfully fertilized too although they do not show 2PN at fertilization check. Previous researches showed that both 0PN- and 1PN-derived blastocysts can result in a successful live birth and healthy infants [12–15]. In addition, whether in the study or in other clinical work, the 0PN- and 1PN-derived blastocysts are only biopsied or transferred when patients do not have any 2PN embryos for transfer. On day 3 (67–69 h postfertilization), the embryos were scored as previously described by the Istanbul consensus [8, 16, 17]: by considering the cell number, fragmentation (%), symmetry, multi-nucleation, vacuoles, and zona pellucida [18]. On days 5/6/7, the expansion of the blastocoel cavity and the number and integrity of both the ICM and TE cells were used to score the blastocysts, just as Gardner and Schoolcrafts' system [19]. However, for ease of blastocyst selection, the ICM and TE grades were further subdivided based on the Gardner classification. Therefore, the ICM and TE were graded as 1–7 rather than A–C in our study. The details of the new scoring criteria are shown in Figs. 1 and 2. We quantified the new scoring criteria and made it easier to be standardized. In the new classification system, ICM diameter was defined as the average value of the longest and shortest diameter. The number of TE cells in the equatorial plane was defined as a main TE scoring criterion. Of note, we still used the assessment parameters of the Gardner classification in our classification system. Therefore, the essence of our classification is still the Gardner classification, and our blastocyst score can be translated to the Gardner classification. For example, 4-7-7 and 4-1-1 equal 4AA and 4CC, respectively. In short, our

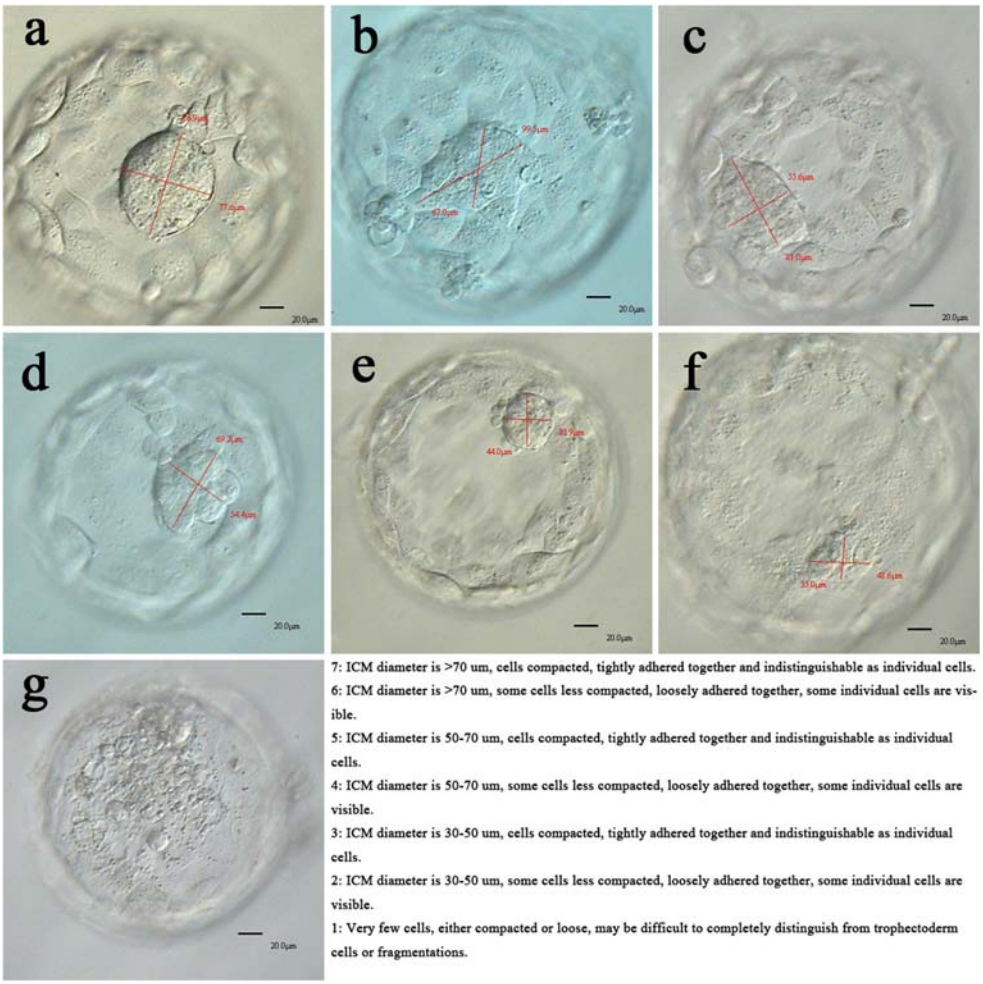

Fig. 1 Photographic example for each ICM grade. The ICM grades of **a**, **b**, **c**, **d**, **e**, **f**, and **g** were 7, 6, 5, 4, 3, 2, and 1, respectively. The ICM diameters of **a**, **b**, **c**, **d**, **e**, and **f** were 82.3, 83.3, 68.3, 61.8, 41.5, and 41.8 μm, respectively. The ICM diameter was defined as the average value of the longest and shortest diameter. In all panels, bar = 20 μm. The details of ICM scoring criteria are as follows: 7, ICM diameter is > 70 μm, cells compacted, tightly adhered together and indistinguishable as individual cells; 6, ICM diameter is > 70 μm, some cells less compacted, loosely adhered together, some individual cells are visible; 5, ICM diameter is 50–70 μm, cells compacted, tightly adhered together and indistinguishable as individual cells; 4, ICM diameter is 50–70 μm, some cells less compacted, loosely adhered together, some individual cells are visible; 3, ICM diameter is 30–50 μm, cells compacted, tightly adhered together and indistinguishable as individual cells; 2, ICM diameter is 30–50 μm, some cells less compacted, loosely adhered together, some individual cells are visible; 1, very few cells, either compacted or loose, may be difficult to completely distinguish from trophectoderm cells or fragmentations

classification is just the subdivisions of the ICM and TE grades based on the Gardner classification. Although the effectiveness of the subdivisions has been validated in our center, it still needs to be validated by more other centers.

In addition, each blastocyst was graded by three embryologists before assignment of the blastocyst grade. To decrease subjective errors, a common prestudy training session was held with the three embryologists. All embryologists had also to pass a scoring consistency test. To control intra-individual variability, only after a blastocyst was graded twice by an operator and the two scores were the same, could the operator determine a grade for the blastocyst. To control inter-individual variability, every operator individually graded all the blastocysts and did not know the blastocyst scores which were given by the other operators before summarizing. That was to say, the three embryologists were mutual blind when they individually graded all the blastocysts. And then, the results of grading (morphology was recorded by photograph) were summarized and inter-operator reproducibility was assessed.

Vitrified-warmed embryo transfer and clinical outcome

The vitrification and warming of the blastocysts was performed using a Cryotop (Kitazato, BioPharma, Shizuoka, Japan) and Vitrification Kit (Kitazato, BioPharma, Shizuoka, Japan) with established methods [20, 21].

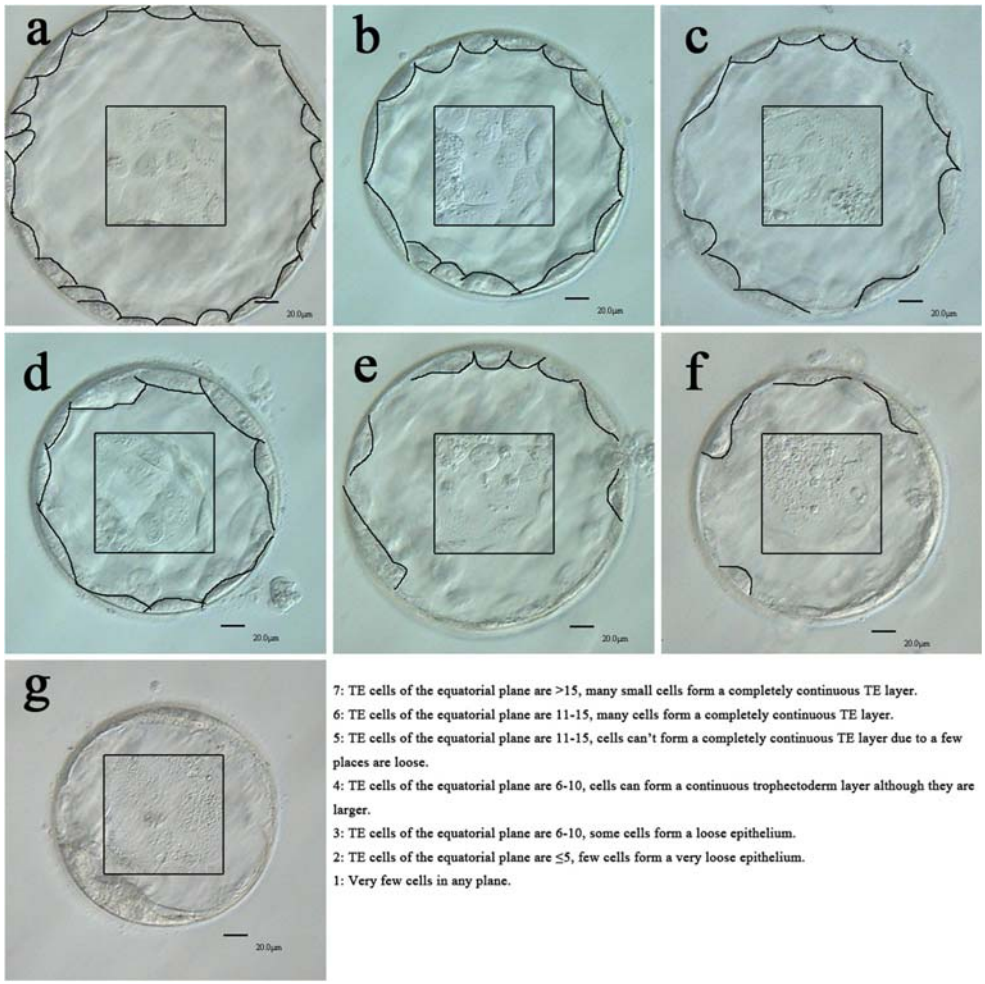

Fig. 2 Photographic example for each TE grade. The TE grades of a, b, c, d, e, f, and g were 7, 6, 5, 4, 3, 2, and 1, respectively. The number of TE cells in the equatorial plane was defined as a main TE scoring criterion. The cell numbers in the equatorial plane of a, b, c, d, e, f, and g were 24, 15, 13, 10, 8, 4, and 0, respectively. The morphologies of TE in other plane were shown by their respective small figures. In all panels, bar = 20 μm. The details of TE scoring criteria are as follows: 7, TE cells of the equatorial plane are > 15, many small cells form a completely continuous TE layer; 6, TE cells of the equatorial plane are 11–15, many cells form a completely continuous TE layer; 5, TE cells of the equatorial plane are 11–15, cells cannot form a completely continuous TE layer due to a few places are loose; 4, TE cells of the equatorial plane are 6–10, cells can form a continuous trophectoderm layer although they are larger; 3, TE cells of the equatorial plane are 6–10, some cells form a loose epithelium; 2, TE cells of the equatorial plane are ≤ 5, few cells form a very loose epithelium; 1, very few cells in any plane

Blastocysts were shrunk by a laser pulse prior to vitrification and then transferred to an equilibration solution for 3–5 min. Subsequently, the blastocysts were transferred to vitrification solution for 1 min and placed into the Cryotop for freezing [7].

To warm to blastocysts, the blastocyst loaded in a cryoloop was immersed in the thawing solution for 1 min. Then, the blastocyst was moved to the dilution solution for 3 min. Finally, the blastocyst was washed in wash solution 1 for 5 min and in wash solution 2 for 1 min [7].

The natural cycle and exogenous steroid replacement cycle were used for endometrial preparation [22]. The warmed blastocysts were cultured for 2 h prior to transfer, and all transfers were single blastocyst transfers. The primary outcome was live birth.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA, version 19.0). The patient and embryo morphological characteristics of the live birth and no live birth group were compared. The data with homogeneous variance were analysed by Student's *t* test, and data with heterogeneous variance were analysed by the Mann–Whitney test. The chi-square test was used for categorical variables. The variables with greater clinical importance and larger variance were selected for further assessment [23]. The correlation between live birth and selected variables was analysed by multivariate logistic regression analysis. The odds ratio (OR) and 95% confidence interval (CI) was also calculated.

Blastocysts in the second part of the study

All blastocysts underwent a TE biopsy at our hospital from June 2016 to September 2018. A total of 4–8 TE cells were gently aspirated with a biopsy pipette followed by a laser-assisted cut [24]. The TE cells were sent to the genetic analysis laboratory for performing whole-genome amplification (WGA) and array-based comparative genomic hybridization (aCGH) (SureScan Microarray Scanner, Agilent, CA, USA). The criteria for biopsy of blastocysts were as follows: [1] expansion status was full expansion, hatching, or hatched; [2] ICM + TE score ≥ 6; [3] whether ICM score or TE score, one of the two scores ≥ 4 was necessary. The ovarian stimulation, embryo culturing and scoring, embryo freezing, embryo thawing, and statistical analysis methods for the second part of the study were the same as those described for the first part of the study, and the cycles involving frozen-thawed oocytes and ICSI were excluded too. There was not any cycle involving gamete or embryo donation in this part.

Results

Part 1: The correlation between the cell number at day 3 and LBR after SVBT

We analysed 1211 SVBT cycles in our center, and 579 of these cycles (47.8%) achieved a live birth. The patients' ages, endometrial preparation methods, and embryo characteristics with respect to the ART outcome are summarized in Table 1. For live birth, significantly lower mean values were found for female age and male age, and a significantly higher mean value was found for the ICM score, TE score, and ICM + TE score. The proportion of 0PN-derived blastocysts in the live birth group was greater than that in the no live birth group (7.25% vs 4.43%), and the proportion of vitrified day 5 blastocysts in the live birth group was greater than that in the no live birth group too (74.96% vs 67.72%). There were no significant differences in the cell number at day 3, embryo fragments at day 3, and expansion or endometrial preparation between the live birth group and no live birth group (Table 1).

Binary logistic regression was performed to control for the cell number at day 3 and the factors with a p value of less than 0.1 in Table 1 (excluding the ICM + TE score because this factor was divided into ICM score and TE score in the model). The 0PN and D5 categories were used as references. The female age, pronuclei number, and TE score had significant effects on live birth. However, for the remaining factors, including the cell number at day 3, the effects were not significant. After adjustment for the female age, male age, pronuclei number, embryo fragments at day 3, ICM, TE, and day of freeze, the adjusted OR for live birth was 1.001 (0.938–1.068) for the cell number at day 3 (Table 2).

Part 2: The correlation between the cell number at day 3 and ER of blastocysts

A total of 864 blastocysts from 292 infertile couples underwent TE biopsy for preimplantation genetic testing for aneuploidies (PGT-A) at our hospital from June 2016 to September 2018, and 858 (99.3%) of these blastocysts had genetic results. Of the blastocysts that had genetic results, 426 (49.7%) were euploid. The patients' ages and embryo characteristics with respect to the genetic results are summarized in Table 3. Regarding euploidy, significantly lower mean values were found for the female age and male age, and significantly higher mean values were found for the cell number at day 3, ICM score, TE score, and ICM + TE score. The proportion of freezing at day 5 among the euploid blastocysts was higher than that among the aneuploid blastocysts (48.83% vs 38.89%). There were no significant differences in the pronuclei number, embryo fragments at day 3, or expansion between euploid blastocysts and aneuploid blastocysts (Table 3).

Binary logistic regression was performed as described in part 1. The D5 category was used as a reference. The ICM score and TE score had significant effects on euploidy, but for the remaining factors, including the cell number at day 3, the effects were not significant. After adjustment for the female age, male age, embryo fragments at day 3, ICM, TE, and day of freeze, the adjusted OR for euploidy was 0.960 (0.866–1.063) for the cell number at day 3 (Table 4).

In addition, the subdivisions based on the Gardner classification were used for blastocyst assessment in the study. The standardization of blastocyst assessment was strictly carried out. Because every grade in our classification was defined as a range value rather than a single value, the inter-operator reproducibility of blastocyst assessment was acceptable. In this study, only 11 blastocyst grades were different among the three embryologists when the embryologists individually graded all the 2075 blastocysts for the first time, the remaining 2064 blastocyst grades were consistent among them. Of these 11 differences, 8 differences lay in ICM grade and 3 differences lay in TE grade. All the differences were due to the vague outlines of ICM or trophectoderm cells. To eliminate these differences, the three embryologists independently reassessed the 11 blastocysts in time. There was only a difference of ICM grade after the 11 blastocyst reassessments. The vague outline of the ICM was discussed and ensured at a high magnification until the ICM grade was consistent among the three embryologists too.

Discussion

In part 1 of our study, we analysed 1211 SVBT cycles to determine the correlation between the cell number at day 3

Table 1 Patient and embryo characteristics of single blastocyst transfer by ART outcome

Characteristics	Live birth ($n = 579$, 47.8%)	No live birth ($n = 632$, 52.2%)	p value
Female age	29.94 ± 4.03	31.04 ± 4.45	$p < 0.001$ [a]
Male age	32.34 ± 5.19	33.22 ± 5.11	$p = 0.003$ [a]
Cell number at day 3	8.64 ± 2.07	8.41 ± 1.88	$p = 0.102$ [b]
Pronuclei number, *n* (%)			
0PN	42 (7.25)	28 (4.43)	
1PN	4 (0.69)	12 (1.90)	
2PN	533 (92.06)	592 (93.67)	$p = 0.022$ [c]
Embryo fragments at day 3, %	2.17 ± 3.81	2.68 ± 4.43	$p = 0.094$ [b]
Expansion, *n* (%)			
No hatching	477 (82.38)	524 (82.91)	
Hatching	86 (14.85)	87 (13.77)	
Hatched	16 (2.76)	21 (3.32)	$p = 0.752$ [c]
Inner cell mass (ICM) score [d]	5.03 ± 0.58	4.96 ± 0.58	$p = 0.026$ [b]
Trophectoderm cells (TE) score [d]	4.87 ± 0.65	4.70 ± 0.69	$p < 0.001$ [b]
ICM + TE score [d]	9.91 ± 0.99	9.66 ± 0.97	$p < 0.001$ [b]
Day of freeze, *n* (%)			
D5	434 (74.96)	428 (67.72)	
D6	139 (24.01)	193 (30.54)	
D7	6 (1.04)	11 (1.74)	$p = 0.018$ [c]
Endometrial preparation			
Natural cycle	183 (31.61)	209 (33.07)	
Exogenous steroid replacement cycle	396 (68.39)	423 (66.93)	$p = 0.587$ [c]

[a] Two-sample *t* test. Values are mean + SD

[b] Two-sample Mann–Whitney test. Values are mean + SD

[c] Pearson's χ^2 test. Values are number (percentage)

[d] ICM and TE grades were 1–7 rather than A–C

and live birth after SVBT. In the logistic regression analysis, after corrections for the effects of the other confounders, the cell number at day 3 had no significant effect on the live birth after SVBT. In part 2 of our study, we analysed 864 blastocysts to determine the correlation between the cell number at day 3 and euploidy of blastocysts. In the logistic regression analysis, after corrections for the effects of the other confounders, the cell number at day 3 had no significant effect on the euploidy of blastocysts. This result indicated that the cell number at day 3 is not an important parameter for blastocyst selection.

Table 2 Multivariable analysis of variables for live birth after single vitrified-warmed blastocyst transfer

Predictors	OR (95% CI)	p value
Female age	1.062 (1.019–1.108)	0.005 [a]
Male age	0.989 (0.956–1.024)	0.535 [a]
Pronuclei number		
0PN	1	
1PN	4.438 (1.257–15.676)	0.021 [b]
2PN	1.719 (1.029–2.872)	0.039 [b]
Cell number at day 3	1.001 (0.938–1.068)	0.968 [a]
Embryo fragments at day 3	1.019 (0.989–1.049)	0.213 [a]
Inner cell mass (ICM) score [c]	0.969 (0.785–1.196)	0.768 [a]
Trophectoderm cells (TE) score [c]	0.763 (0.631–0.922)	0.005 [a]
Day of freeze		
D5	1	
D6	1.219 (0.922–1.610)	0.164 [b]
D7	1.407 (0.498–3.969)	0.519 [b]

[a] p value of each variable's overall effects after adjusting for the other variables

[b] p value between each variable's subgroups and reference group

[c] ICM and TE grades were 1–7 rather than A–C

However, previous studies showed that an excessively high or low embryo cleavage rate has a negative impact on the embryo chromosomal constitution [10, 25]. It seems that the cell number at day 3 has an effect on embryo euploidy, and this conclusion conflicts with our conclusion. The different conclusions are due to the different embryo biopsy times between the two previous studies and our study. Embryo biopsy was performed at cleavage stage in the two previous studies and was performed at blastocyst stage in our study. The different conclusions can be drawn based on the different biopsy times; the reasons are as follows: First, a result of cleavage-stage biopsy is easily affected by embryo mosaicism due to sampling only one or two blastomeres. The few blastomeres also can decrease the accuracy of the result [26]. However, blastocyst biopsy can avoid this limitation. Second, although the results of cleavage-stage biopsy indicated that the "abnormal" cell number at day 3 increases the aneuploid rate of

Table 3 Patients' ages and embryo characteristics of preimplantation genetic testing for aneuploidies by blastocyst ploidy status

Characteristics	Euploidy ($n = 426$, 49.7%)	Aneuploidy ($n = 432$, 50.3%)	p value
Female age	33.01 ± 4.66	34.24 ± 5.06	$p < 0.001$ [a]
Male age	34.96 ± 5.59	36.47 ± 6.09	$p < 0.001$ [a]
Cell number at day 3	8.18 ± 1.47	7.91 ± 1.48	$p = 0.015$ [b]
Pronuclei number, *n* (%)			
0PN	25 (5.87)	26 (6.02)	
1PN	7 (1.64)	11 (2.55)	
2PN	394 (92.49)	395 (91.44)	$p = 0.648$ [c]
Embryo fragments at day 3, %	2.39 ± 4.15	3.04 ± 4.79	$p = 0.067$ [b]
Expansion, *n* (%)			
No hatching	58 (13.62)	80 (18.52)	
Hatching	308 (72.30)	287 (66.44)	
Hatched	60 (14.08)	65 (15.05)	$p = 0.110$ [c]
Inner cell mass (ICM) score [d]	4.57 ± 0.58	4.46 ± 0.56	$p = 0.002$ [b]
Trophectoderm cells (TE) score [d]	4.35 ± 0.59	4.16 ± 0.60	$p < 0.001$ [b]
ICM + TE score [d]	8.92 ± 0.78	8.62 ± 0.79	$p < 0.001$ [b]
Day of freeze, *n* (%)			
D5	208 (48.83)	168 (38.89)	
D6	210 (49.30)	251 (58.10)	
D7	8 (1.88)	13 (3.01)	$p = 0.011$ [c]

[a] Two-sample *t* test. Values are mean + SD

[b] Two-sample Mann–Whitney test. Values are mean +SD

[c] Pearson's χ^2 test. Values are number (percentage)

[d] ICM and TE grades were 1–7 rather than A–C

cleavage-stage embryo [10, 25], an aneuploid cleavage-stage embryo can be eliminated during blastocyst formation [27]. Therefore, once blastocyst formation is successfully completed, the blastocysts with the "abnormal" cell number at day 3 may not have a higher aneuploidy rate. Thus, blastocyst biopsy, rather than cleavage-stage biopsy, is the best way to determine the correlation between the cell number at day 3 and euploidy of blastocysts. Because of the two reasons, the results of cleavage-stage biopsy have some important limitations to be used in blastocyst selection, although they can be used in cleavage-stage embryo selection. And our study avoided these limitations by using blastocyst biopsy. In addition, one of the previous studies had a conjecture that a blastocyst with faster cleavage rate may be more likely to be aneuploid than a blastocyst with "normal" cleavage rate [25]. However, this conjecture was made based on the data of cleavage-stage biopsy. It had some limitations too and the authors also stated the limitations in their paper. The limitations are as follows: (1) The testing results of the same embryo may be different between cleavage-stage biopsy and blastocyst biopsy due to embryo mosaicism. Aneuploid blastomeres can be excluded as cell fragmentations during blastocyst formation. (2) Cleavage-stage biopsy would potentially affect embryo developmental progression to the blastocyst stage [28]. It would skew results and affect the validity of the conjecture. To avoid these limitations of the conjecture, the authors suggested that blastocyst biopsy would be performed. In our study, embryo biopsy was performed at blastocyst stage.

Table 4 Multivariable analysis of variables for euploidy of blastocysts

Predictors	OR (95% CI)	p value
Female age	1.033 (0.982–1.088)	0.208 [a]
Male age	1.021 (0.979–1.066)	0.332 [a]
Cell number at day 3	0.960 (0.866–1.063)	0.430 [a]
Embryo fragments at day 3	1.023 (0.990–1.057)	0.168 [a]
Inner cell mass (ICM) score [c]	0.730 (0.564–0.944)	0.017 [a]
Trophectoderm cells (TE) score [c]	0.597 (0.464–0.767)	< 0.001 [a]
Day of freeze		
D5	1	
D6	1.152 (0.850–1.561)	0.362 [b]
D7	1.330 (0.516–3.428)	0.555 [b]

[a] p value of each variable's overall effects after adjusting for the other variables

[b] p value between each variable's subgroups and reference group

[c] ICM and TE grades were 1–7 rather than A–C

Therefore, our conclusion that the cell number at day 3 is not a strong predictor of the ER of blastocyst may be more acceptable, although the cell number at day 3 has an effect on the ER of cleavage-stage embryo [8]. In fact, the data of the previous study also showed that there was no significant difference in aneuploid rate between blastocysts with "normal" cleavage rate and those with faster cleavage rate (54% vs 62%; $p = 0.096$) [25]. This result was consistent with that of our study.

In addition, in part 1 of our study, female age and TE score were strong predictors of live birth after SVBT. This result was consistent with those of previous reports [3, 23, 29, 30]. In part 2, blastocysts were obtained from the patients who underwent PGT-A. Therefore, the infertile couples comprised mostly patients of advanced age. Although there were some young patients in this part, their embryos might have a higher aneuploid rate than those of the general population due to repeat implantation failure or idiopathic recurrent miscarriage. It might be the reason why the age of patients was not a strong predictor of blastocyst euploidy in our logistic regression analysis. The ICM score and TE score were strong predictors of ER. This result was consistent with those of previous reports [24, 31, 32].

There was no significant difference in the pronuclei number between euploid blastocysts and aneuploid blastocysts. We also divided the blastocysts into three groups according to the pronuclei number. The ERs of 0PN-, 1PN-, and 2PN-derived blastocysts were 49.02% (25/51), 38.89% (7/18), and 49.94% (394/789), respectively. There were no significant differences in the ER among the three groups (χ^2 test, $p = 0.648$). This result was consistent with that of previous report [33]. It indicated that both 0PN- and 1PN-derived blastocysts can be used for transfer when these blastocysts are obtained by conventional IVF. However, the pronuclei number was a strong predictor of live birth after SVBT in the logistic regression analysis of part 1. It indicated that 0PN-, 1PN-, and 2PN-derived blastocysts have different developmental potentials although they have no significant differences in ER. The LBRs of the 0PN-, 1PN-, and 2PN-derived blastocysts also had a significant difference (60.00% vs 25.00% vs 47.38%, χ^2 test, $p = 0.022$). The 0PN-derived blastocysts had the highest LBR might be due to the 0PN category had the highest proportion of day-5 blastocyst (78.57%). In contrast, the 1PN-derived blastocyst category had the lowest proportion of day-5 blastocyst (62.50%). The proportion of day-5 blastocyst in the 2PN-derived blastocyst category was 70.84%. Day-5 blastocysts have a higher LBR than day-6 blastocysts [34].

Because this was a retrospective study, certain variables could not be collected. We recognized there were some important limitations in this study. First, in part 1, fresh embryo transfer was not performed in any patients, it was mainly due to the risks of ovarian hyperstimulation syndrome and intrauterine environment unsuitable for embryo implantation. Therefore, we did not include the other patients who could be performed a fresh embryo transfer. And the average age of the included patients was lower because the patients with a risk of ovarian hyperstimulation syndrome usually were young. Secondly, we did not include the ICSI cycles. Thirdly, in analysis of part 1, we only included blastocysts which were transferred. And in analysis of part 2, we only included blastocysts which met freezing criteria. All the limitations might lead to a population selection bias. Therefore, further studies should be done with enlarged patients and blastocysts samples.

Conclusion

In the population of our study, we conducted a detailed analysis of the correlation among the cell number at day 3, LBR after SVBT, and ER of blastocysts. The results showed that the cell number at day 3 is not a strong predictor of the LBR when the vitrified-warmed blastocysts obtained by conventional IVF are transferred into young patients. In addition, the cell number at day 3 is not a strong predictor of ER of IVF-derived blastocysts too. Therefore, when we select a vitrified-warmed blastocyst obtained by conventional IVF for a young patient, we do not have to focus on the cell number at day 3.

Authors' roles All of the authors (Zhiren Liu, Mingting Jiang, Linyun He, and Yun Liu) made substantial contributions to the conception of the study and to the design or acquisition of the data, as well as to the analysis and interpretation of the data. Z.L, M.J, and Y.L drafted the article or revised it critically for important intellectual content. All of the authors gave final approval of the version to be published.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study, formal consent is not required.

Informed consent Informed consent was obtained from all individual participants included in the study.

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