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Effect of blastomere cell number on ART outcome of fresh single day 3 embryo transfer

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Abstract

Purpose Explore the effect of blastomere cell number on ART outcome of fresh embryo transfer on day 3.

Methods Retrospective analysis of 540 fresh single day 3 embryo transfer cycles at the Reproductive Center of the Third Affiliated Hospital of Guangzhou Medical University from January 1, 2018 to October 31, 2022. Patients were divided into 5–6 cell group ($n=55$), 7–9 cell group ($n=457$), and ≥ 10 cell group($n=28$) based on the number of blastomeres. Single factor analysis of variance and Pearson's chi square test were used to compare the basic data, cycle information, pregnancy outcome and neonatal outcome. Univariate logistic regression was used to correct for confounding factors and analyze the influencing factors of pregnancy outcome.

Results The positive HCG rate were 20%, 43%, 25% for the 5–6-cell, 7–9 cell and ≥ 10 cell groups respectively, with statistically significant differences ($P<0.001$). The clinical pregnancy rate was 18%, 42%,21%, respectively ($P<0.001$). The live birth rates were 13%, 34%,21% with P-value less than 0.05 which is statistically significant. In order to exclude the influence of confounding factors, multivariable logistic regression analysis was performed, and the outcomes were consistent with previous findings. There were no significant differences found in neonatal outcome between groups ($P>0.05$).

Conclusion The results suggested that intermediate cleaving embryos (7–9 cell) still presents the highest clinical potential. Fast and slow cleaving embryos are not conducive to the ART outcome.

Keywords Blastomere number, Fresh single day 3 embryo transfer, ART outcome

Introduction

In recent years, the prevalence of infertility in China has been on a steady rise, attributed to various factors such as delayed marriage and childbirth, unhealthy lifestyles, and deteriorating environmental pollution [1]. With the advent of Assisted Reproductive Technology (ART), clinical ovulation induction protocols have undergone continuous development and optimization, resulting in the refinement of embryo culture techniques. Consequently, artificial Insemination (AI), in vitro fertilization—embryo transfer (IVF-ET) and intracytoplasmic sperm injection- embryo transfer (ICSI-ET) have emerged as efficient therapeutic modalities for addressing infertility issues. However, the widespread adoption of ART technology has led to a concurrent surge in the incidence of multiple pregnancies [2]. Multiple pregnancy represents

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a significant adverse outcome associated with ART, significantly elevating the risk of premature delivery for pregnant women and elevating the incidence of birth defects among newborns [3]. Furthermore, it imposes a substantial economic burden on both patients and society. Therefore, optimizing pregnancy outcomes while effectively mitigating multiple birth rates has emerged as a crucial breakthrough in the realm of reproductive medicine.

The selection of high-quality embryos with high implantation potential is pivotal for the success of ART [4] and serves as a crucial juncture in ART procedures. Over the past decade there has been an increasing trend to extending culture from cleavage-stage to blastocyst transfer. Presently, numerous reproductive centers primarily adopt a blastocyst transplantation strategy, which improves the pregnancy rates while mitigating the risk of multiple pregnancies. However, laboratory culture conditions and the embryo itself makes the higher failure rate of blastocyst culture. Blastocyst transfer is associated with increased number of cycles with no embryos to transfer. In addition, this approach has led to a notable elevation in the secondary sex ratio (SSR) [5]. The Istanbul Consensus Workshop's expert consensus in 2011 [6] advocated for the use of elective single embryo transfer (e-SET) as a reliable and efficient transfer strategy to minimize multiple pregnancies. Furthermore, additional studies have demonstrated that fresh D3 single embryo transfer can effectively reduce the incidence of multiple pregnancies and enhance pregnancy outcomes [7–9]. Consequently, the transfer of a single day 3 cleavage embryo possesses certain advantages.

The Istanbul Consensus Workshop [6] has identified the cleavage stage of the embryo, reaching 8 blastomeres on the third day post-fertilization, as the optimal developmental speed for D3 embryos. Tomic and colleagues [10] observed that among D3 embryo transfers, 8-cell embryos exhibit the highest implantation potential following fertilization. Racowsky et al. [11] reported in the SART CORS (Society for Assisted Reproductive Technology Results Reporting System) that there exists a positive correlation between survival rate and an increase in cell count up to 8, with the highest survival rate being observed in D3 cleavage-stage embryos. However, some scholars [12] argue that human blastocyst morphological quality is significantly improved in embryos classified as fast on day 3 ($\geq$10 cells). Other studies have demonstrated equivalent clinical outcomes between over ten-cell good embryo transfers on day three and those of eight-cell embryos [13]. These findings challenge current embryological dogma.

In addition, despite having lower blastocyst formation rates, slow cleaving embryos demonstrate robust developmental potential upon transplantation, successfully implanting and resulting in live births [14–16]. Consequently, in scenarios where the patient lacks a high-quality cleavage embryo, we opt to transfer embryos with lower scores, inclusive of those exhibiting either excessively rapid or slower development. The aim of this study was to ascertain the optimal rate of embryonic cleavage development that leads to the most favorable ART outcomes, ultimately serving as a clinical basis for refining embryo culture methods and single embryo transfer practices.

Materials and methods

Study design and patients

This study is a retrospective review of subjects undergoing fresh single day 3 embryo transfer at Guangzhou Medical University Affiliated Third Hospital Reproductive Center from January 1, 2018 to October 31, 2022. This study was approved by the ethics committee of the Third Affiliated Hospital of Guangzhou Medical University. Data contain sensitive patient information will be made available on request. Written informed consent was obtained from all the participants.

Exclusion criteria: FET cycle; fresh blastocyst transfer; fresh D3 transfer with 2 or more embryos; fresh two-step transfer embryos (one embryo was transferred on the third day after oocyte retrieval and another on the fifth day); cases with missing data in the electronic medical record.

A total of 540 fresh transfer cycles were included in this study. Based on the number of blastomeres on day 3, they were divided into 3 groups: 5–6 cells, 7–9 cells, and $\geq$10 cell.

Review of diagnosis and treatment

ART procedures are executed in accordance with our hospital's standardized operating protocols. Tailored ovulation induction regimens are employed based on the unique circumstances of individual patients, including the long protocol, ultra-long protocol, short protocol, follicular phase long protocol, antagonist protocol, micro-stimulation protocol, and natural cycle protocol. These protocols are selected based on factors such as the patient's age, body mass index (BMI), antral follicle count (AFC), and anti-Mullerian hormone (AMH) levels. Egg retrieval is performed via the vaginal route, approximately 35–36 h after the injection of human chorionic gonadotropin (hCG). Routine in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) is performed based on semen parameters and previous fertilization outcomes. For conventional IVF, mobile sperm is fertilized in a fertilization medium (G-IVF PLUS, Vitrolife, Gothenburg, Sweden) by combining it with an oocyte

complex. In the case of ICSI, oocytes are stripped 2–3 h after egg retrieval, and sperm microinjection is carried out 5–6 h later. Fertilization is assessed approximately 16 h post-insemination/injection and is confirmed by the presence of two pronuclei. The embryos are then incubated in a controlled environment (K-MINC-1000; Cook Medical, Bloomington, IN, USA), maintained at 37°C with 6% CO_2 and 5% O_2. The cleavage-stage embryos are cultured using G-1 PLUS medium (Vitrolife, Gothenburg, Sweden). During the luteal phase, vaginal microprogesterone (400 mg/day) is administered from the day of ovarian puncture.

D3 embryo quality assessment

The assessment of embryo quality at the cleavage stage occurred approximately 68 h after insemination/injection. This evaluation encompassed the quantification of blastomeres, determination of the proportion of fragments, and measurement of blastomere sizes, and the best quality embryo is selected for transfer. If the patient has high-quality embryo (high-quality embryos were defined as 7–9 cells with a uniform blastomere size and without fragment), we chose preferentially from the 7–9 cell group. In scenarios where the patient lacks a high-quality cleavage embryo, we opt to transfer an available embryo (a blastomere count of ≥ 5, a fragment proportion ≤ 20%, and a uniform blastomere size).

Follow-up

Clinical outcome

The level of human chorionic gonadotropin (β-hCG) was measured by blood sampling 14 days after embryo transfer, and β-hCG ≥ 10 IU/L was defined as positive. After transplantation, vaginal B-ultrasonography was performed, and the pregnancy was diagnosed as clinical pregnancy if the pregnancy sac and the original cardiac tube beat. Early abortion before 12 weeks of gestation; Late abortion at 13 to 27 weeks of gestation.

Neonatal outcome

Neonatal sex, height, weight, and malformation were tracked.

Outcome parameters

The primary clinical outcomes encompassed the HCG positivity rate, clinical pregnancy rate, early abortion rate, late abortion rate, ectopic pregnancy rate, and live birth rate. Furthermore, the major perinatal outcomes were gender distribution, average height and weight, ultra-low weight (≤ 2500 g), overweight (≥ 4000 g), and the proportion of congenital malformations.

Statistical analysis

Data analysis was performed using SPSS 25.0 statistical software. Continuous variables were expressed as mean ± standard deviation ($\bar{x} \pm s$), and between-group comparisons were made using one-way analysis of variance. For categorical variables, data were presented as frequency and percentage n (%), and between-group comparisons were made using Pearson's chi-square test. Logistic regression analysis was conducted with HCG positivity rate, clinical pregnancy rate, early miscarriage rate, late miscarriage rate, and ectopic pregnancy rate as dependent variables. The data were presented as odds ratios (OR) with confidence intervals (CI), and confounding factors were adjusted to accurately assess the independent impact of the quality of D3 cleavage-stage embryos on clinical outcomes. The significance level for hypothesis testing was set as two-sided, with $P < 0.05$ indicating statistical significance.

Results

A total of 540 subjects were identified, fresh single day 3 embryo were transferred and grouped according to the blastomere number, which were categorized into 5–6 cell group,7–9 cell group, ≥ 10 cell group. The flow diagram of the study is shown in Fig. 1. Baseline demographics of the study cohort are presented in Table 1. Embryos were stratified based on number of cells on day 3, with 10.19% of embryos with 5–6 cells,and 84.62% of embryos with 7–9 cells, and 5.19% of embryos with ≥ 10 cells. All baseline characteristics were similar among groups, except for the mean number of the BMI.

Table 2 demonstrates the cycle characteristics stratified by day 3 cell number. There was no significant different between the groups for comparison of ovulation regimen, days of ovulation, type of endothelium on ET day, Fragmentation percentage, type of fertilization. Comparison among the three groups revealed significant differences in total amount of Gn (IU), thickness of endothelium (mm) on ET day, No. of oocytes retrieved, blastomere symmetry.

Pregnancy outcomes according to blastomere number are detailed in Table 3. The rate of live birth rate (LBR) was 13.0%, 34% and 21% for the 5–6-cell, 7–9-cell and ≥ 10-cell groups, respectively. LBR varied significantly among groups ($P = 0.015$). Likewise, the positive hCG test and clinical pregnancy rates also show a similar trend with different blastomere number ($P = 0.001$). While miscarriage rate (both early abortion rate and late abortion rate) and multiple births rate were not statistically significant with *P*-value greater than 0.05. Only one case of gestational hypertension occurred in the 7–9 cell group, and there were no pregnancy complications in

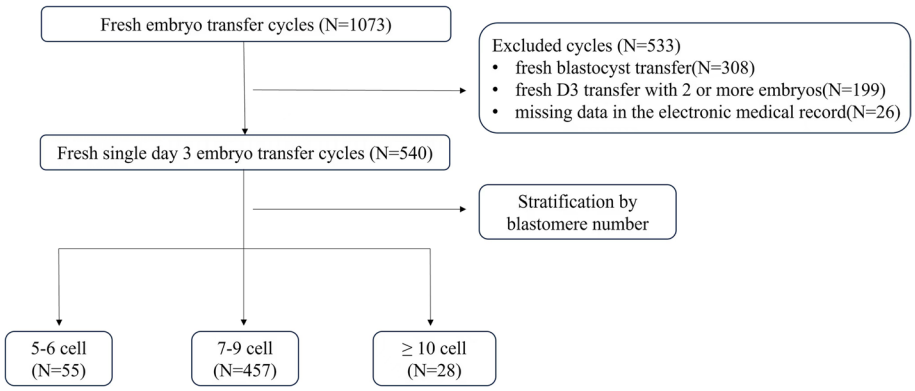

Fig. 1 Flowchart showing data selection process for analysis

Table 1 Demographic characteristics of fresh single day 3 embryo transfer cycles stratified by the blastomere number

Number of blastomere	5-6 cell	7-9 cell	≥10 cell	*P*-value
Number of cycles	55	457	28	
Maternal age (years)	34.24±5.10	33.31±4.84	32.61±5.53	0.155
Maternal BMI (kg/m^2)	23.50±3.55	22.22±3.22	22.20±2.83	0.032
Type of infertility, n (%)				0.221
Primary infertility	23(41.80)	185(40.50)	16(57.10)	
Secondary infertility	32(58.20)	272(59.50)	12(42.90)	
Infertility causes, n (%)				0.113
Female reason	26(47.30)	261(57.10)	9(32.10)	
Male reason	12(21.8)	82(17.9)	8(28.6)	
Reasons of both sides	17(30.9)	110(24.1)	10(35.7)	
Unknown reason	0(0.00)	4(0.90)	1(3.60)	
AMH (ng/ml)	3.62±3.25	3.86±3.02	2.68±1.65	0.071
E2 (pmol/ml)	140.48±115.96	155.94±179.56	203.60±207.70	0.264
FSH (mIU/ml)	6.31±2.09	6.09±2.21	5.73±1.38	0.631
LH (mIU/ml)	4.00±3.47	3.83±2.97	5.27±7.18	0.055

the other two groups, the difference was not statistically significant.

In order to exclude the influence of confounding factors, multivariable logistic regression analysis was performed, and the outcomes were consistent with previous findings. The variables included female age, female BMI, type of infertility, causes of infertility, stimulation protocols, thickness of endothelium on ET day, type of endothelium on ET day, fragmentation percentage, blastomere symmetry and type of fertilization. As shown in Table 4, after controlling for potential confounding factors, the 5–6 cell group was associated with lower LBR compared with the 7–9 cell group (aOR 0.377, 95% CI 0.156–0.914; $P=0.031$). Similarly, the live birth rate was significantly lower in patients transferred with ≥ 10 cells embryos (aOR 0.3, 95% CI 0.101–0.896,$P=0.031$). The positive hCG test and clinical pregnancy rates also show a similar trend with different blastomere number.

Neonatal outcomes are described by Table 5 descriptive statistics. There were a total of 77 male and 93 female fetuses, of which the significant difference between the sexes of the fetuses in each group was not significant ($P>0.05$). There was no significant difference in weight, height and fetus week number (Table 3) between the groups (P value >0.05).

Discussion

Due to various factors, the incidence of infertility in Chinese women has shown a trend of increasing year by year in recent years [17]. Assisted reproduction technology (ART), as a new and effective assisted reproductive technology, has been widely used in the treatment

Table 2 Cycle characteristics of fresh single day 3 embryo transfer cycles stratified by the blastomere number

Number of blastomere	5–6 cell	7–9 cell	≥ 10 cell	*P*-value
Number of cycles	55	457	28	
Ovulation regimen, n (%)				0.683
Long protocol	39(70.9)	321(70.2)	23(82.1)	
Antitackiness agent	12(21.8)	112(24.5)	4(14.3)	
Special type	4(7.3)	24(5.3)	1(3.6)	
Days of ovulation (d)	11.98 ± 3.19	11.11 ± 2.87	11.00 ± 3.06	0.150
Total of Gn (IU)	2765.00 ± 1352.28	2247.29 ± 1174.52	2393.30 ± 1031.86	**0.010**
Thickness of endothelium on ET day(mm)	9.93 ± 2.22	10.30 ± 2.20	11.20 ± 2.08	**0.037**
Type of endothelium on ET day, n (%)				0.217
A	32(58.2)	295(64.6)	14(50.0)	
B	23(41.8)	162(35.4)	14(50.0)	
No. of oocytes retrieved	6.78 ± 4.60	9.31 ± 4.71	6.79 ± 3.69	**< 0.001**
Fragmentation percentage, n (%)				0.217
< 10%	19(34.5)	252(55.1)	9(32.1)	
10%-20%	36(65.5)	205(44.9)	19(67.9)	
Blastomere symmetry, n (%)				**< 0.001**
0	20(36.4)	368(80.5)	6(21.4)	
+	35(63.6)	89(19.5)	22(78.6)	
Type of fertilization, n (%)				0.141
IVF	45(81.8)	353(77.2)	17(60.7)	
ICSI	9(16.4)	98(21.4)	10(35.7)	
IVF + ICSI	1(1.8)	6(1.3)	1(3.6)	

Table 3 Pregnancy outcomes stratified by the blastomere number

Number of blastomere	5–6 cell	7–9 cell	≥ 10 cell	*P*-value
Number of cycles	55	457	28	
HCG positive rate	0.20(11/55)	0.43(198/457)	0.25(7/28)	**0.001**
Clinical pregnancy rate	0.18(10/55)	0.42(191/457)	0.21(6/28)	**0.001**
Ectopic pregnancy rate	0(0/10)	0.02(3/191)	0(0/6)	**< 0.001**
Early abortion rate	0.20 (2/10)	0.13 (24/191)	0 (0/6)	0.505
Late abortion rate	0.10 (1/10)	0.03 (5/191)	0 (0/6)	0.363
Live birth rate	0.13 (7/55)	0.34 (157/457)	0.21 (6/28)	**0.015**
Rate of multiple births	0 (0/10)	0.01 (1/191)	0 (0/6)	0.959
Fetus week number (week)	38.32 ± 1.17	38.60 ± 1.70	38.27 ± 1.88	0.856
Pregnancy complications (N)	0	1	0	0.959

Table 4 Multivariable logistic regression analysis of potential factors associated with outcomes

Pregnancy outcomes		5–6 cell	7–9 cell	≥ 10 cell
HCG positive rate	aOR, 95% CI	0.429 (0.202,0.911)	reference	0.265 (0.096,0.733)
	P-value	**0.028**		**0.010**
Clinical pregnancy rate	aOR, 95% CI	0.417 (0.192,0.905)	reference	0.212 (0.072,0.627)
	P-value	**0.027**		**0.005**
Live birth rate	aOR, 95% CI	0.377 (0.156,0.9140)	reference	0.300 (0.101,0.896)
	P-value	**0.031**		**0.031**

Table 5 Neonatal outcomes stratified by the blastomere number

Number of blastomere	5-6cell	7–9 cell	≥ 10cell	*P*-value
Gender, n (%)				0.351
Male	3(42.90)	73(46.50)	1(16.70)	
Female	4(57.10)	84(53.50)	5(83.30)	
Singleton birth height(cm), mean ± SD	50.43 ± 1.27	49.38 ± 2.53	49.17 ± 2.48	0.256
Singleton birth weight(kg), mean ± SD	3142.86 ± 475.59	3054.44 ± 659.65	2930.00 ± 642.03	0.777
Low-Weight n(%) < 2500 g, n (%)	0(0.00)	15(9.60)	1(16.70)	0.577
Excess weight n(%) ≥ 4000 g, n (%)	0(0.00)	5(3.20)	0(0.00)	0.808
Malformation, n(%)	0(0.00)	2(1.30)	0(0.00)	0.920

of infertility. Choosing the optimal embryos for transfer to achieve high pregnancy rates without increasing multiple pregnancy rates is the key to ART. IVF/ICSI mostly adopts fresh cleavage embryos on the third day for transplantation [18]. The quality of embryos at the cleavage stage on day 3 of fresh transplantation has a significant impact on pregnancy outcomes. Currently, embryo quality evaluation is mainly based on morphological evaluation [19, 20]. The number of blastomeres and grade of fragmentation are important indicators for assessing embryo quality [21]. Moreover, Nagy ZP et al. claimed that polarization of nucleolus precursor bodies in both pronuclei is as reliable marker of implantation as embryo morphology on day 3 [22]. Subsequent literature reports indicate that oocytes exert a significant influence on embryo quality [23]. Furthermore, a study conducted by Jing Fu and his colleagues [24] have revealed that early cleavage serves as a crucial metric in the assessment of implanted embryo quality. However, there is still controversy regarding which factor should be prioritized when selecting embryos for transplantation in clinical settings. Some studies suggest that the number of embryos is more suitable as an indicator for clinical embryo selection [12], while Haibin Zhao et al. maintain that the proportion of embryo fragments is a superior proxy to the number of cleavage-stage embryos [13, 25]. Based on the consensus proposed by the Chinese Society of Reproductive Medicine in collaboration with multiple reproductive centers [26], our study categorized the blastomeres into three groups: 5–6 cell group, 7–9 cell group, and ≥ 10 cell group, using the number of blastomeres as the grouping criterion.

The reason why do embryos with more or less than 7–9 cells have poorer pregnancy outcomes remains unclear. It is speculated that embryos at the 8-cell stage and faster developing cleavage stage embryos exhibit comparable rates of blastocyst formation and implantation potential, which are both superior to slow-cleaving embryos [6, 11, 13]. Luna et al. even suggested that compared to 8-cell embryos, faster embryos (> 10 cells) on day 3 may form higher quality blastocysts, while increased blastomere number in cleavage-stage embryos is associated with higher aneuploidy [12, 27, 28]. However, there have been relatively few studies examining the pregnancy outcomes and neonatal outcomes of different types of embryos after transplantation on the third day. After comparing and analyzing the three groups of data and adjusting for confounding factors, we found that the HCG positive rate, clinical pregnancy rate, and live birth rate in the 7–9 cell group were significantly higher than those in the other two groups, and the differences were statistically significant, which suggests that blastomeres with 7–9 cells are more suitable for clinical embryo transfer than those with 5–6 cells or ≥ 10 cells. Additionally, we undertook a rigorous statistical analysis to assess neonatal outcomes and discovered no noteworthy disparities in gender, stature, weight, or congenital malformations among newborns resulting from embryo transfers involving varying blastomere counts. This indicates that the number of blastomeres utilized in embryo transfer does not significantly influence the gender, height, weight, or occurrence of malformations in newborns.

To our knowledge, only limited studies have been conducted to investigate the impact of day 3 cell number on clinical outcomes of cleavage-stage transfer cycles. The present study, aiming to overcome the drawbacks of previously published studies, re-analyzed the pregnancy outcomes. The novelty of this study is that it explores for the first time the effect of blastomere number on neonatal outcomes. Furthermore, multiple regression models were established to control for a variety of confounding factors.

This study has some certain limitations, mainly reflected in its being a single-center study that lacks sufficient diversity to broadly represent different environments. Additionally, in the subgroup analysis of neonatal outcomes, the sample size of some subgroups was relatively small, which may have affected the stability and reliability

of the relevant statistical results. Despite these limitations, the study provides a foundation for further research and discussion. Therefore, when interpreting and applying the findings of this study, it is necessary to fully consider these limitations. Future studies can further validate and extend the conclusions of this study through multi-center collaboration and increased sample size.

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Authors' contributions
YLM and LL contributed to the conception and design of the study and the drafting of the article. LT, CYS and YXH contributed to collect clinical date. HYH and YQS contributed to the statistical analysis. LL revised the manuscript. LL was responsible for approval of the final version. All authors reviewed the manuscript.

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Availability of data and materials
The data sets in this study cannot be publicly available because of involving the patient privacy but are available from the corresponding author on reasonable request.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This study was approved by the ethics committee of the Third Affiliated Hospital of Guangzhou Medical University. Each patient has signed an informed consent on obtaining and analyzing their clinical data prior to the initiation of IVF/ICSI-ET treatment.

Consent for publication
The author confirms that the study described has not been published before, that its publication has been approved by all co-authors and that its publication has been approved (tacitly or explicitly) by the responsible authorities at the institution where the study was carried out.

Competing interests
The authors declare no competing interests.

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