Does Blastocyst Morphology Influence Live Birth Rate after Frozen-thawed Single Euploid Blastocyst Transfer?

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Research

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Abstract

Objective

To determine whether the morphologic parameters of euploid blastocyst influence the live birth rate (LBR) following single frozen-thawed embryo transfer (FET) cycles?

Methods

A retrospective cohort analysis involving autologous single FET cycles after next generation sequencing (NGS) based preimplantation genetic testing for aneuploidy (PGT-A) by a large in vitro fertilization (IVF) center that was performed from June 2017 to September 2019. Women were divided into three age groups (< 30, 30–34 and ≥ 35 years old). The primary outcome measure was LBR. Outcomes were compared between different blastocyst quality (Good, Average and Poor), inner cell mass (ICM) grade (A and B), and trophectoderm (TE) grade (A, B and C).

Results

A total of 232 FET cycles were included, the live birth rate was 48.28%. In the youngest group (< 30 years old, n = 86), LBR were compared between cycles with various blastocyst quality (72.22% for good quality, 54.55% for average quality and 34.78% for poor quality; \( P = 0.019 \)), ICM grade (70.59% for grade A and 42.03% for grade B; \( P = 0.035 \)) and TE grade (85.71% for grade A, 57.58% for grade B and 34.78 for grade C; \( P = 0.015 \)). Nevertheless, either in the 30–34 years group (n = 99) or in the oldest group (≥ 35 years, n = 47), LBR were also comparable between these subgroups, no significant difference was showed in blastocyst morphologic parameters and LBR \( (P > 0.05) \). Furthermore, in the similarly graded euploid blastocysts, there was also no statistical significance in LBR among different age subgroups \( (P > 0.05) \).

Conclusions

In women ≥ 30 years old, euploid blastocyst quality was not associated with the LBR in FET cycles, highlights the development competence of poor-quality euploid blastocysts.

Introduction

The development of assisted reproductive technology (ART) has greatly helped many infertile couples realize their wishes to be parents in the past few decades, while embryo arrested development and early spontaneous abortion are still difficult problems in the treatment of infertility. Studies have shown that the high incidence of embryo aneuploidy is one of the important factors affecting IVF-ET success \(^{[1,2]}\), which manifested as about 50% of embryos occurring errors during gametogenesis and early mitotic divisions throughout their preimplantation development. It is not morphologically distinguishable from
euploid embryos at cleavage or blastocyst stages[3]. Furthermore, with the increase of work pressure and the acceleration of life rhythm, more and more women choose to have children late. However, they will face a lower fecundability and higher miscarriage rates with age gradually increase[4]. This condition is attributed to the rapid increase of aneuploidy rates in women ≥ 34 years old[5]. Morphologic assessment is a relatively common method of prioritizing IVF embryos for transfer at present. As we all know, blastocysts morphology alone cannot accurately evaluate aneuploidy status and that about half of embryos with better morphological scores were eventually detected as aneuploidy[6].

With the advances in molecular biology, and the aim of ART is always to obtain a healthy singleton live birth, since then the three-generation IVF technique PGT-A developed and improved the success of ART by allows patients to avoid the transfer of aneuploid blastocysts to achieving live birth[7]. The current technology used for PGT-A testing is based on NGS, which is done using Polymerase Chain Reaction (PCR) amplification and then the results of sequencing are distinguished from normal and abnormal amounts of DNA[8]. Nowadays, PGT-A is mainly applied to the following population: such as elderly women, repeated implantation failure, recurrent spontaneous abortion and severe oligoasthenospermia and teratozoospermia[9].

Several researchers have studied the relationship between embryo quality and live birth rate in non-PGT-A cycles; while there are few studies concerned about the benefits of PGT-A on clinical outcomes in ART. Minasi et al. showed embryos with good quality have a statistically significantly higher euploidy rate and implantation rate (IR) than those with poor quality[6]. Nevertheless, Anderson et al. concluded that once a single euploid embryo is transferred, high levels of implantation and live birth success are attained independent of embryo quality[10]. Considering that the effect of embryo quality on blastocysts ploidy and pregnancy outcomes. Therefore, the objective of our study was to identify whether the morphologic parameters of euploid blastocyst influence LBR in single FET cycle, which is helpful for doctors to select embryos to transfer and provide consultation for patients in clinical practice.

**Materials And Methods**

**Study design and patients**

This retrospective cohort study was performed at the Reproductive Medicine Center of the Third Affiliated Hospital of Zhengzhou University between June 2017 and September 2019. Women who undergone first autologous PGT-A and followed by single euploid FET cycles were enrolled. Finally, only 232 cycles were found to be eligible for inclusion in the data analysis, as shown in Fig 1. This study was approved by the Institutional Review Board of our hospital.

**Ovarian stimulation protocol**

Each female patient underwent a standard ovarian stimulation, trigger of oocyte maturation, oocyte retrieval, fertilization, embryo culture and transfer as we previously described[11]. The Gonadotropin
(Gonal-F, Merck Serono, Switzerland) started injection from the second or third day of the menstrual cycle, dosage (150-300IU) was adjusted based on patient's age, basal antral follicle count (AFC), body mass index (BMI), basal follicle stimulating hormone (FSH) and ovarian reserve. The response to stimulation was assessed by performing transvaginal ultrasounds and measuring serum estradiol levels. GnRH antagonist (Cetrotide, Merck Serono, Switzerland) was usually used for pituitary suppression. GnRH antagonist 0.25 mg (Dophereline, Ipsen Pharma Biotech, France) was used to trigger the final oocyte maturation. Ultrasound guided oocyte retrieval was performed 33-36 hours after the trigger.

**Laboratory protocols**

Blastocyst evaluation was performed prior to embryo biopsy. Blastocysts were graded according to the Gardner and Schoolcraft grading system, and the score was dependent on blastocyst expansion, ICM development and trophectoderm TE appearance\(^\text{[12]}\). The degree of expansion included the following six grades: (1) a nonexpanded embryo with the blastocele filling <50%; (2) the blastocele filling >50% of the embryo; (3) a full blastocyst with a blastocele filling the embryo; (4) an expanded blastocyst with a blastocele volume larger than that of the full blastocyst, with a thinning zona; (5) a hatching blastocyst with the TE starting to herniate through the zona; and (6) a hatched blastocyst, with the blastocyst completely escaping from the zona. In our center, for blastocysts with an expansion score \(\geq 4\), the development of the ICM and TE was then evaluated and the ICM grade should at least B. The ICM was graded as follows: (A) tightly packed, with many cells; (B) loosely gathered, with several cells; and (C) very few cells. The three TE grades were (A) many cells forming a cohesive epithelium, (B) few cells establishing a loose epithelium and (C) very few large cells. The quality of the blastocyst was grouped into three categories based on ICM and TE scoring: good quality: AA, AB and BA; average quality: BB; and poor quality: AC and BC. Embryo grading was performed by the same team of four highly trained embryologists and each with five years of experience, which minimized the difference in human judgment. Then the embryos were biopsied on day 5 or day 6 based on the time of blastulation. The zona pellucida was perforated by use of a Saturn laser system (Research Instruments, Singapore) to opening of 6–9 mm, and a biopsy pipette was used to aspirate 3–5 herniated TE cells. Then the washed TE cells were placed in 0.2-mL PCR tubes containing 5 μL phosphate-buffered saline solution (PBS). All selected embryos were screened for 24 chromosome aneuploidy with NGS, as described in Zimmerman et al\(^\text{[13]}\). Finally, three different outcomes were considered after the PGT-A testing: euploid and aneuploid and mosaic. After the biopsy, the blastocyst were vitrificated using Cryotop® (Kitazato Corporation, Shizuoka, Japan)\(^\text{[14]}\).

**Endometrial preparation**

Embryos that were screened by NGS to be euploid were transferred in FET cycles. In general, women with regular ovulatory cycles underwent natural FET cycles and the artificial cycles were applied for women with irregular menses, ovulation dysfunction or thinner endometrial thickness. After five days ovulation and when endometrial thickness was \(\geq 7\) mm, which were all monitored by vaginal ultrasound,
only single frozen-thawed euploid blastocyst was transferred and provided for conventional luteal support.

**Outcome measures and statistical analysis**

All statistical results were calculated with SPSS 25.0 statistical software (IBM, United States). LBR after the transfer of euploid embryos are we mainly discussed measure in this study. LBR was defined as the number of live births divided by the sum of embryos transferred cycles included in the cohort. All cycles were divided into three groups according to the women's age (<30, 30-34 and ≥35 years old). The outcomes measure and the baseline demographic characteristics were compared among the three groups.

Categorical variables were compared with the Chi-square ($X^2$) and Fisher's exact tests. Continuous variables were tested for normality. They were expressed as mean ± standard deviation, and parametric data were compared using the analysis of variance (ANOVA) test. $P<0.05$ was considered to be statistically significant.

**Results**

A total of 232 cycles followed by FET were included in this study. The TE biopsy with NGS was performed in 608 blastocysts. A total of 516 (98.10%) blastocysts with conclusive results were included for analysis, in which 248 (48.06%) were euploid and the other were aneuploid (51.94%), while 10 (1.90%) blastocysts produced no results (Fig. 1). The LBR was 48.28%.

The association between women's age and LBRs was evaluated in Fig. 2. LBR reached highest in women aged 30 years old and then declined gradually with women's age.

The exposure measure of this study was maternal age. All cycles were categorized into three groups: <30 years old (n = 88), 30–34 years old (n = 99), ≥ 35 years old (n = 47). The demographic characteristics of patients are summarized in Table 1. There was no significant difference in BMI, FSH, endometrial thickness on transfer day, infertility diagnosis and day of TE biopsy among the three age groups. The proportion of secondary infertility was highest in the oldest age group ($P<0.001$).
Table 1
Characteristic of women who underwent PGT-A cycles.

| Characteristic                              | < 30 (n = 86) | 30–34 (n = 99) | ≥ 35 (n = 47) | P value |
|--------------------------------------------|--------------|----------------|--------------|---------|
| Age(years)                                 | 26.79±2.09   | 31.52±1.27     | 38.74±3.23   | < 0.001 |
| BMI(kg/m²)                                 | 24.30±2.94   | 24.07±2.92     | 24.20±3.02   | 0.639   |
| FSH(IU/L)                                  | 6.37±2.53    | 6.52±2.02      | 7.14±2.47    | 0.598   |
| AMH(ng/ml)                                 | 5.35±3.97    | 5.36±3.95      | 4.99±3.83    | 0.866   |
| Endometrial thickness on transfer day(mm)  | 9.22±1.37    | 8.85±1.69      | 8.97±1.65    | 0.273   |
| Type of infertility(%)                     | < 0.001      |
| Primary                                    | 44(51.16)    | 29(29.29)      | 4(8.51)      |
| Secondary                                  | 42(48.84)    | 70(70.71)      | 43(91.49)    |
| Infertility diagnosis(%)                   | 0.587        |
| Female factor                              | 24(27.91)    | 40(40.40)      | 17(36.17)    |
| Male factor                                | 28(32.56)    | 31(31.31)      | 13(27.66)    |
| Combined factor                            | 25(29.07)    | 18(18.18)      | 12(25.53)    |
| Unexplained                                | 9(10.47)     | 10(10.10)      | 5(10.64)     |
| FET endometrial preparation(%)             | 0.697        |
| Natural cycles                             | 34(39.53)    | 63(63.63)      | 32(68.09)    |
| Artificial cycles                          | 52(60.47)    | 36(36.37)      | 15(31.91)    |
| Day of TE biopsy(%)                        | 0.379        |
| Day 5                                      | 45(52.33)    | 62(62.62)      | 28(59.57)    |
| Day 6                                      | 41(47.67)    | 37(37.38)      | 19(40.42)    |

As shown in Table 2, the LBR of different morphologic parameters related to blastocysts quality were comparable between all age groups (Table 2). In the youngest age group (< 30 years), the prevalence of live birth was 72.22% for good quality, 54.55% for average quality and 34.78% for poor quality (P = 0.019). Nevertheless, the blastocyst quality did not affect LBR in the other two age groups. In women aged 30–34 years old, LBRs was 50% for good quality, 51.52% for average quality and 45.45% for poor quality (P = 0.870). Similarly, the oldest patients (≥ 35 years) had comparable LBR, ranging from 57.14–42.11% (P = 0.671).
Table 2
Live birth rate in women of different age groups.

| Age       | < 30 (n = 86) | 30–34 (n = 99) | ≥ 35 (n = 47) |
|-----------|---------------|---------------|--------------|
| Embryo quality |               |               |              |
| Good (n = 47) | 72.22         | 50.00         | 42.86        |
| Average (n = 76) | 54.55         | 51.52         | 57.14        |
| Poor (n = 109) | 34.78         | 45.45         | 42.11        |
| P value     | 0.019         | 0.870         | 0.671        |
| Day of TE biopsy |           |               |              |
| Day 5 (n = 135) | 62.22         | 51.61         | 57.14        |
| Day 6 (n = 97) | 31.71         | 43.24         | 36.84        |
| P value     | 0.005         | 0.533         | 0.238        |
| ICM grade   |               |               |              |
| A (n = 42)  | 70.59         | 35.29         | 62.50        |
| B (n = 190) | 42.03         | 51.22         | 46.15        |
| P value     | 0.035         | 0.291         | 0.261        |
| TE grade    |               |               |              |
| A (n = 26)  | 85.71         | 60.00         | 50.00        |
| B (n = 97)  | 57.58         | 47.50         | 54.17        |
| C (n = 109) | 34.78         | 45.45         | 42.11        |
| P value     | 0.015         | 0.634         | 0.821        |

When cycles were stratified according to the ICM grade before the day of TE biopsy, cycles in which ICM were graded A were comparable with cycles in which ICM were graded B: 70.59% vs 42.03% (P = 0.035) in women younger than 30 years old. However, 51.22% vs 35.29% (P = 0.291) in women aged 30–34 years old and 62.50% vs 46.15% (P = 0.261) in women aged 35 years or older.

Likewise, the effect of different TE grades also had a relationship with LBR in youngest women (< 30 years old), which ranged from 85.71–34.78% (P = 0.015). But in women aged 30 years or older, TE grade did not influence LBRs, which ranged from 60–45.45% in 30–34 age group (P = 0.634) and 54.17–42.11% in patients aged ≥ 35 years old (P = 0.821).

The influence of similarly graded embryo quality on LBR in different age subgroups were also evaluated in Fig. 3. However, the difference in LBR of similarly graded embryo quality did not reach statistical
significance \( (P > 0.05) \).

**Discussion**

Our findings investigated the effect of euploid blastocyst morphology on LBR following FET cycles. Our results demonstrate that euploid blastocyst morphology irrespective of LBR in women aged 30 years or older. The current study also showed gradually decline with age in LBR in women after the age of 30 years old. We further indicated that when cycles were stratified according to the similarly graded euploid embryo quality of different age subgroups, blastocysts quality also do not influence the LBR.

In general, the maternal age is a key factor determining the possibility of pregnancy following ART\textsuperscript{[15]}. Most importantly, the reason for age-related decline in reproductive ability is contributed to the increase of aneuploidy. It seems reasonable that we speculated the effect of maternal age on pregnancy outcome is eliminated after PGT-A. However, a literature reported that in women \( \leq 35 \) years the chance of conception increased higher than those older 35 years old\textsuperscript{[1]}\textsuperscript{[1]}\textsuperscript{[1]}\textsuperscript{[1]}\textsuperscript{[1]}. Likewise, contrary to our expectation, we also found that LBR rose slowly with women's age and reached highest in women aged 30 years old and then started to gradually decline. We assume that other than aneuploidy, there are other age-related factors affecting fecundity. Given that we also taking blastocysts morphologic parameters into consideration in this study. To our surprise, we found the morphological scoring of euploid blastocysts is related to LBR in the population under 30 years old. However, the difference is not obvious in women age 30 years or older.

Several previous studies have reported that even among the euploid embryos, traditional morphologic scoring has been still used to assess blastocysts development potential and forecast pregnancy outcome\textsuperscript{[16, 17]}. Irian et al.\textsuperscript{[18]} confirmed that good quality euploid embryos were associated with a higher IR and LBR than poor quality euploid embryos. In their another retrospective study, they also concluded that better morphologic scores embryos yield a higher ongoing pregnancy rate compared with lower morphologic grading euploid blastocysts\textsuperscript{[19]}. Consistent with the literature, our results confirm that the morphology of the euploidy blastocyst is very important and helpful for embryo selection, especially in women younger than 30 years old. Suggesting we might pay more attention to blastocyst morphology when selecting embryos in the patients under 30 years old. A recent research demonstrated that LBR is not affected by embryo quality in advanced women once PGT-A has been performed. The authors highlight the development potential of poor quality euploid blastocysts\textsuperscript{[20]}. This is also concordant with our results. So, poor quality embryos should not be discarded, which can reduce the transfer cycles and economic burden of patients. At the same time, we should combine time-lapse microscopy, metabonomics and protein profiles to comprehensively evaluate the quality of embryos and screen out the embryos with the most developmental potential. While Capalbo et al.\textsuperscript{[21]} found that none of the morphologic parameters provides additional valuable information for PGT-A to select the best developmental embryos for transfer. The only included small sample size, therefore this conclusion may not represent the general population.
The traditional blastocyst grading system including three morphologic parameters: the degree of blastocoel expansion, ICM and TE. Efforts were also made to relates to the implantation potential of euploid blastocysts. There are conflicting data regarding the predictive value of morphology parameter on pregnancy outcomes. Several researchers have reported that ICM morphology can statistically significantly predict LBR \cite{22,23}, because ICM is differentiated into fetal, so ICM grade should theoretically be the most important morphologic feature influencing transfer outcomes. However, recent publications in human have shown that TE quality should be corrected with viability\cite{24,25}. This may due to TE become into the placenta, and healthy trophectoderm is required to have the capacity to invade the endometrium to initiate the complex process of implantation and to maintain normal pregnancy progress. At the same time, some researchers noted that the degree of blastocoel expansion were statistically significant predictors of clinical pregnancy rate (PR) and LBR. The very small sample size of euploid blastocysts with blastocoel expansion grades 5 and 6, so we did not perform the predictive value of blastocoel expansion on LBR in our study. We found that in the younger population, embryos with a better grading of ICM and TE are associated with a higher LBR compared with embryos with lower morphology grading. Therefore, we think both ICM and TE are good predictors for evaluating the LBR. Although we found that in single euploid FET, the TE and ICM grade were not corrected with the LBR in older women, this is consistent with our main results.

When stratifying by age, there is no significant difference in the LBR between different age subgroups in the similarly graded euploid blastocysts. This shows the female age has little effect on the live birth success in the same graded euploid embryos. This is also the significance of taking morphological parameters as an important index for embryos selection in our study.

The strength of our research are as follows. First, all embryos and cycle were processed at a single reproductive center. Second, embryo scoring was conducted by the same team of four highly trained embryologists and each with five years of experience. Third, strict inclusion and exclusion criteria for statistical study and we only transfer single euploid embryo that underwent first autologous PGT-A-testing, this may eliminate factors which we have known can influence our outcomes. The present study also has some limitations. First, the retrospective analysis design is subject to inherent confounding and bias that cannot be neglected. Second, if more than one euploid embryo is available for transfer, euploid blastocysts with the good quality are usually preferred when we selected blastocysts. Thus, this may cause selection bias. Third, the number of cases in each embryo quality category were relatively small. Large prospective or sample size analysis are required to validate our current findings in the future studies.

**Conclusion**

In general, this study provides guidance for reproductive center medical worker that the commonly used morphologic parameters of blastocysts assessment are also suitable for PGT-A cycles, especially improving the selection of euploid embryos among women younger than 30 years old. Furthermore, in clinical practice, we can provide consulting services for older than 30 years patients if there are no good
quality euploid embryos are available for transfer, poor quality euploid embryo are also an option, because they will produce similar LBR.

**Abbreviations**

LBR: Live birth rate; FET: Frozen-thawed embryo transfer; NGS:Next generation sequencing; PGT-A: Preimplantation genetic testing for aneuploidy; IVF: In vitro fertilization; ICM: Inner cell mass; TE: Trophectoderm; IR: Implantation rate; ART: Assisted reproductive technology; PCR: Polymerase Chain Reaction; AFC: Antral follicle count; BMI: Body mass index; FSH: Follicle stimulating hormone.

**Declarations**

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**Authors’ contributions**

Study design: Na Li, Hua Lou. Data acquisition and analysis: Bingnan Ren, Yuchao Zhang and Yulin Du. Drafting of the manuscript and interpretation: Na Li, Hua Lou. Revision of the manuscript: Yichun Guan. All authors read and approved the final manuscript.

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**Availability of data and materials**

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

The study was approved by the Institutional Review Board and Ethics Committee of the Third Affiliated Hospital of Zhengzhou University.

**Competing interests**

The authors declare that they have no competing interests.

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