Impact of embryo biopsy stage on implantation potential in thalassemias: a propensity score matching study

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Research

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Abstract

Objective: To investigate the impact of biopsy stage on implantation potential of frozen-warmed blastocysts in pre-implantation genetic testing (PGT) cycles for those with thalassemias.

Design: Retrospective paired cohort study using propensity score matching (PSM). A time-to-event analysis of Fine-Gray's analysis was used to compare cumulative live birth rate (CLBR).

Setting: Single university-affiliated center .

Patient(s): A total of 439 PGT couples were included who underwent PGT for thalassemias between 2015 and 2017.

Intervention(s): Two biopsy strategies were evaluated; cleavage-stage biopsy (CB) and blastocyst-stage biopsy (BB).

Main Outcome Measure(s): Implantation rate and CLBR.

Result(s): A total of 202 ovum pick-up (OPU) cycles from Group CB and 237 OPU cycles from Group BB were included. PSM was conducted for two groups covering characteristics on the OPU cycle and the first frozen-warmed blastocyst transfer (FWBT) cycle , resulting in 140 matched pairs. The implantation rate was significantly lower in the CB group (41.0%, 30.3%, 20.4% in 1st, 2nd and 3rd FWBT cycle respectively, weighted mean 34.2%) than those in the BB group (51.8%, 43.5%, and 40.5% (weighted mean 48.1%), respectively; at $P = 0.044, 0.047, 0.039$). This was a 28.9% relative reduction ($P = 0.002$). The CLBR across three FWBT cycles was significantly higher in the BB group compared with the CB group (80.0% vs. 62.1%, $P = 0.032$), while the likelihood of LBR was 1.21-fold higher after regression adjustment.

Conclusion(s): Cleavage-stage biopsy may partially affect the implantation potential and CLBRs of frozen-warmed blastocysts, compared with blastocyst stage biopsy.

Introduction

Pre-implantation genetic testing (PGT) has been conducted for couples at high risk of transmitting genetic defects to their offspring. One critical component of PGT procedures is the embryo biopsy. Since the evolution of extended culture conditions, blastocyst-stage biopsy (BB) has emerged as an increasingly used methodology, accounting for roughly 60% of biopsy strategies according to the latest ESHRE PGT consortium data collections (1, 2). However, cleavage-stage biopsy remains the preferred biopsy method in some situations as it affords an opportunity for a fresh embryo transfer(3), and takes advantage of PGT for mitochondrial disorders since samples from blastomeres may represent whole post-natal heteroplasmy(4).

In order to attain embryonic genetic material, both techniques involve removing cells from embryos in clinical practice; either as one or two blastomeres from CB or three to ten trophectoderm cells (TCs) from

BB(5). Regarding the former technique, the limited cell number, high mosaicism rate, and pluripotent property of cells could compromise its accuracy, and may exert a detrimental effect on embryo viability and progressed delivery rate(6, 7). Additionally, the CB approach raises concerns about the increasing incidence of low birth weight offspring and impaired cognitive and/or motor development in early childhood (8). As for the BB technique, this appears to be a more technically robust procedure where more can be removed during biopsy process but there is less cellular content and certainty with respect to extra-embryonic cell loss. However, the range of cells obtained has not been precisely defined for BB. Besides, blastocysts with different qualities possess different trophectoderm-layer elasticity (9), which provides a challenge with respect to manipulation and culture conditions. Asynchronous development of blastocysts also increases labor workload. Owing to the asynchronous expansion degree, blastocysts that develop at an extremely slow rate of development might not be considered as competent for biopsy, hence, there is the possibility of a high wastage rate(10, 11). In addition to the afore-mentioned technical issues, concerns of safety also remain. More recently, Taskin and co-workers demonstrated that BB was associated with reduced likelihood of full-term in mice models, even though blastocyst viability was not affected(8). This was the situation when paralleled with the longer duration of incubation beyond embryonic genomic activation. BB has also been reported to have adverse effects on placental functions e.g., increasing risk for gestational hypertension and a 3-fold increase in odds for pre-eclampsia(12, 13). In some cases, especially for those with mitochondrial DNA diseases, although PGT on cleavage-stage embryos seems robust, it might impact upon blastocyst development.

Previous studies have already compared the two biopsy strategies with respect to clinical outcomes. However, the results were controversial. Some trials reported a more compromised implantation potential of biopsied embryos and an inferior clinical pregnancy rate per cycle after CB was performed(6, 14, 15), while others found no statistically significant differences between CB and BB(16, 17). It is worth noting that bias may exist in previous studies e.g. prolonged blastocyst culture and/or vitrification only performed in BB embryos but not in CB embryos that were compared (with the advantage of fresh ET). Besides, there are other common queries such as, e.g. whether biopsy stage affects cumulative live birth rate, and the data for questions such as this are either unclear or not yet reported.

Since results from previous studies have been conflicting, further investigations using a more optimized design or larger sample size are required. However, to the best of our knowledge, no randomized controlled trial comparing the impact of these two biopsy stages on blastocyst development and implantation potential has been published to date.

In recent years, our laboratory gradually changed biopsy strategy from cleavage stage to blastocyst stage. During the transition period, we performed cleavage-stage biopsy for α -thalassemia with South-East Asia (SEA) deletion, and blastocyst stage biopsy for β -thalassemia to reduce the possibility of misdiagnosis. The purpose of this study was to analyze the impact of biopsy stage on the implantation potential over this period of time. Propensity score matching (PSM) analysis, which has the power to mimic randomized assignment for the purpose of minimizing imbalances in observational studies, was utilized for subgroup matching in the present study. Under such adjusted conditions, we investigated the

cumulative live birth rate (CLBR) of three consecutive frozen-warmed cycles after deferred blastocyst transfer between CB and BB groups in PGT for thalassemias.

Materials And Methods

Patients

This retrospective study included data from thalassemia carriers who received PGT and underwent FWBT between June 2014 and December 2015 in the Reproductive Medical Center of the First Affiliated Hospital of Sun-Yat San University, Guangzhou, China. Our center has a history of more than 15 years using fluorescent gap PCR analysis for detection of α -thalassemia with SEA deletion, and PCR-reverse dot blot (RDB) hybridization analysis for β -thalassemia diagnosis. As previously stated, couples were initially divided into two groups according to the mutation types of thalassemias: couples with α -thalassemiaSEA genotype receiving CB (Group CB), and couples with one of the 16 common β -thalassemia mutations receiving BB (Group BB). Embryos were frozen by vitrification following blastocyst culture in Group CB, or just after TE biopsy for Group BB.

There had to be at least one blastocyst diagnosed as a normo-homozygote or heterozygote (unaffected blastocysts jointly), which survived for transfer after warming. Confounders which may have had an adverse impact on implantation were excluded. These included female age ≥ 35 , intrauterine adhesion, uterine malformation/scar, unexplained recurrent miscarriage, immune disorders (systemic lupus erythematosus, antiphospholipid syndrome), and thyroid dysfunction. Finally, 202 cycles in Group CB and 237 cycles in Group BB met our criteria.

All study subjects were required to accept an initial consultation at both the Reproductive Medical Center and its affiliated Genetic Laboratory to acquire information on all aspects of the COS and PGT procedures.

Embryo Culture and Biopsy

In Group CB, oocytes after ICSI were cultured in G1 medium (Vitrolife, Sweden) for 3 days under 6% CO₂, 5% O₂, and 89% N₂ in a COOK incubator (Bloomington, IN, USA). Embryos that had at least five or more cells with <50% of fragmentation (qualified cleavage-stage embryos, QCSEs) early on day 3 after oocyte retrieval were placed into 20 μ l Ca²⁺/Mg²⁺ free medium under mineral oil (SAGE, BioPharma, US) and subjected to biopsy following zona opening using a non-contact laser (OctaxShot™ laser system). A single blastomere from each embryo was removed by a sampling micropipette. Biopsied blastomeres were placed immediately in RNase and DNase-free PCR tubes containing 10-20 μ l of PCR-grade water. Biopsied embryos were rinsed thoroughly and transferred gently into G2 medium (Vitrolife, Sweden) until day 5 or 6 after oocyte retrieval for extended culture. Obtained blastocysts graded better than 3BC/3CB on day 5 or 6 were vitrified using the Cryotop (KitazatoBiopharma Co. Ltd., Shizuoka, Japan) method.

In Group BB, embryos were directly cultured to the blastocyst stage in sequential media (G1 and G2, Vitrolife, Goteborg, Sweden) under the same incubation conditions. Blastocysts graded better than 3BC/3CB on day 5 or day 6 were selected for biopsy. A zona hole was drilled by laser on the morning of day 5. TE biopsy was performed when TE cells were herniated from the zona hole. Typically, 5-10 TE cells were aspirated according to two steps described by Kokkali *et al.*(16). The protocol of cryopreservation in this group was the same as blastocysts from Group CB as previously mentioned.

Genetic testing

Fluorescent gap PCR analysis was utilized for detecting α -thalassemia SEA deletion, while PCR-reverse dot blot (RDB) hybridization analysis was applied for β -thalassemia diagnosis, as shown in our previously published papers. Sixteen β -thalassemia mutations, including CD41-42, IVS-2nt 654, CD17, -28, CD71/72, -29, β E, CD43, -32, -30, Int, CD14/15, CD27/28, CD1/1, CD1/5, and CD31, were covered. The primer sequences and product sizes are shown in Table 1 and all PCRs were performed following the same reaction system and PCR conditions as described previously(18). No chromosome screening was performed in either group.

Blastocyst warming and transfer

The blastocyst warming procedure in both groups was conducted as previously described (19). Only those blastocysts that survived warming were considered for transfer. According to the Gardner-Scoring system based on the degree of expansion and the quality of the inner cell mass (ICM) and of the TE cells, blastocysts were morphologically categorized into 3 grades: excellent (expansion degree of 5-6, with both ICM and TE quality of Grade B or better), good (expansion degree of 5-6, with either ICM or TE quality of Grade C; 4BB or better), and average (graded 3BC/3BC or better but not qualified in the foregoing groups) (20). No more than two, usually single unaffected blastocysts, were transferred using a COOK catheter (Guardia™) 3-4 hours after warming in both groups (frozen-warmed blastocyst transfer, FWBT).

Tailored natural ovulation cycle (NC) or hormone replacement treatment protocol (HRT) was formulated for endometrial preparation. In HRT cycles, oral estrogen (Progynova, BAYER) was prescribed with an initiation dose of 2 mg, twice daily. Doses of estrogen were increased gradually according to the development of endometrium. When endometrium thickness reached 8 mm, a 60 mg dose of intramuscular progesterone (Zhejiang Xianju Pharmaceutical Co., Ltd.) was given. Alternatively, a dose of 90 mg micronized vaginal progesterone bio-adhesive gel preparation (Crinone 8%, Merck Serono) combined with 10 mg oral progesterone tablets, twice daily (Dupbastone, Abbott) was administered. Blastocysts were warmed and transferred on the sixth day of progesterone administration, or 5 days after ovulation.

Clinical outcomes

We compared baseline characteristics of the two groups in COS cycles, including maternal age, BMI, and levels of basal FSH, LH, and E₂, embryo morphology on Day 3 and Day 5/6, and diagnostic results. Pivotal parameters affecting embryo implantation were analyzed. These specifically covered the endometrium preparation protocols, interval time from the OPU day of stimulation cycle to the embryo transfer (ET) day of subsequent first FWBT cycle, blastocysts grades after warming, and average number of embryo transferred, amongst other factors. Outcomes in the successive 1st, 2nd and 3rd FWBT cycles were followed-up.

A preclinical pregnancy was defined as two consecutive positive rising (10 IU/ml) serum β -HCG levels, measured 12 days after transfer day and with an interval of 3 days. A clinical pregnancy was defined as fetal viable heartbeats detected by a transvaginal ultrasound scan at least 6 weeks after embryo transfer. A clinical miscarriage was defined as a spontaneous pregnancy loss (cycles of biochemical pregnancy and multiple pregnancy reduction were excluded) before 28 weeks of gestation. Follow-up data on pregnancies and deliveries were collected via phone contacts (records in delivery file used for birth certificate or discharge records). The implantation rate (detected gestational sacs divided by the total embryos transferred) was taken as a primary outcome measure, whereas the cumulative live birth rate (CLBR, number of patients with first live birth that had been achieved from the first to the current cycle divided by the initial number of patients, irrespective of the duration of gestation and additional live births) was the end-point of the study.

Statistical Analysis

The statistical analysis was performed using Statistical Package for Social Science, version 23.0 (SPSS Inc., IBM corp). Continuous variables with normal distribution were given as mean $\pm$ standard deviation (SD) and tested using the Student's *t*-test. Otherwise the Mann-Whitney U test was utilized, as demonstrated by the Kolmogorov-Smirnov test. Statistical analysis of contingency tables was performed with the use of Pearson's chi-square analysis, or, if any expected frequency was ≤ 4 , by a two-tailed Fisher exact test.

To reduce selection bias, confounding factors covering maternal age at OPU, basal hormone levels, history of miscarriage, stimulation protocols, numbers of oocytes retrieved, parameters of the first FWBT as the endometrium preparation protocols, blastocyst grades after warming, number of transferred embryos, endometrial thickness, and levels of E₂ and P on the day of transfer, were set as the PSM independents. The PSM module was utilized to calculate propensity score by using the nearest neighbor matching as logistic regression, and 1:1 patient matching proportion was performed. The Hofemer-lemeshow test was used to assess the goodness of categorical variables fit. The calipers value was set as 0.2. The Fine and Gray's test was applied to perform a competing risk analysis between CB and BB considering comparison of CLBR, where early pregnancy loss was considered a competing risk to the

outcome live birth in our study. This analysis was conducted using Stata (version 15.0, StataCorp LLC). All analysis was two-sided and a P value of <0.05 was considered to be statistically significant.

Results

The baseline characteristics with regards to mean maternal age, body mass index (BMI) and basal endocrine level (FSH, LH, E_2) were comparable between the two groups. However, 54.4% of Group BB patients had no miscarriage history, while 60.4% Group CB patients miscarried at least once before ($P = 0.005$).

A total of 1880 cleavage-stage embryos were biopsied in the 202 OPU cycles, and 1089 blastocysts were formed after biopsy in Group CB, leading to a qualified blastocyst formation rate (QBFR) of 57.9% (1089/1880). Of the obtained blastocysts, 1005 (92.3%) had a corresponding diagnosis. The transferrable blastocysts (blastocysts diagnosed as normo-homozygote or heterozygote genotype) per cycle was 3.04 ± 1.95 .

In Group BB, biopsy was performed on 1205 blastocysts, which developed from 1992 QCSEs in the 237 OPU cycles. The QBFR was 60.5% (1205/1992), of which 1126 (93.4%) provided thalassemia genotype results. The transferrable blastocysts per cycle was 3.40 ± 1.32 , with no statistically significant difference compared to that of Group CB ($P = 0.152$).

The survival rates of vitrified blastocysts were similar between Group CB and BB (97.7% vs 98.1%) (Table 2). There were no statistically significant differences between the two groups regarding the endometrium preparation protocol, blastocysts grade after warming, average number transferred, progesterone level on embryo transfer day, and subsequent luteal support protocol. There were statistically significant differences noted for the remaining variables which were associated with implantation potential and these included history of miscarriage, endometrial thickness and estradiol levels on ET day (see tables 2 and 3). After PSM with maternal age, blastocyst grade, number of embryos transferred, ET-day progesterone levels and the biased correlated factors (history of miscarriage, endometrial thickness, and estradiol level on ET day) of the first FWET cycle, 140 paired cycles were matched from two groups (Table 4). The baseline characteristics were well-balanced between the two sub-groups (Table 5).

Clinical outcomes of the three consecutive FWBT cycles from matching subgroups were further compared. Significantly lower implantation rates were observed in Group CB (41.0%, 30.3%, and 20.4% in successive 1st, 2nd and 3rd FWBT cycles, weighted mean of 34.2%), compared with those in Group BB (51.8%, 43.5%, and 40.5%, respectively; weighted mean 48.1%: $P = 0.044, 0.047, 0.039$), with a 28.9% relative reduction on the weighted average [$1-(34.2\%/48.1\%)$] ($P = 0.002$).

A higher ongoing pregnancy rate (47.9%) and a lower clinical miscarriage rate (7.7%) in Group BB were detected in the 1st FWBT cycle, compared to those in Group CB (37.3%, 11.6%, respectively; $P = 0.042, 0.032$). However, there was a tendency to the former group of ongoing pregnancy rate in both groups detected in the 2nd and 3rd FWBT cycles.

The CLBR was 62.1% (87/140) for the Group CB, which was significantly lower than 80.0% (112/140) observed in Group BB (Figures 1a and 1b). In Grey's competing risk analysis, a dominantly higher CLBR was observed in Group BB compared with Group CB with an increment of 17.9% ($P = 0.032$). Comparisons were illustrated in detail in Table 6.

Furthermore, we tried to evaluate the comparative "take-home baby" rate considering different biopsy strategies. A multivariable analysis of influential factors associated with the live birth rate in the initial group without PSM in the first FWBT was analyzed using the Fine-Grey model (initially, 202 CB cycles and 237 BB cycles were included). Independent factors, such as maternal age, PSM, E_2 levels on OPU day, number of oocytes, variables in the ET day (covering endometrial thickness, the number and quality of embryos, and levels of E_2 , P_4 as well), were adjusted for. Only biopsy method and endometrium thickness on ET day were screened out as being independently decisive. BB presented with a 1.21-fold higher likelihood of live birth compared with CB (95%CI 1.06-4.35, $P = 0.041$). With respect to endometrium being thicker than 5 mm as reference, 5-8 mm, and >8 mm, respectively were associated with a 2.31- and 3.52-fold higher possibility of a live birth compared to those less than the reference threshold ($P = 0.005$, < 0.001, respectively) (Table 7).

Discussion

Although cleavage stage is still adopted for PGT biopsy, application of BB is increasingly being used, with cases per year and number of centers offering this option soaring. CB and BB each have their own advantages and limitations. Here, we mainly compared the implantation competence of embryos after CB and BB. Our results showed that BB was associated with a higher embryonic implantation potential compared to CB.

Theoretically, removing blastomeres is more invasive than TE removal. Two factors have predominantly been considered to be important in inferior implantation capacity after CB: (i) less tolerance to cell loss. When removing a blastomere or two from a cleavage stage embryo, cell numbers may be reduced by 12.5-33%. The study by Anick *et al.* showed that 8-cell embryos with 2-blastomere removal for biopsy have a 40% lower implantation potential compared to 8-cell embryos with only 1-blastomere removal(7). As for BB, although it is possible that up to 10 TE cells can be retrieved, it merely comprises for 3.3–10% of content of an expanded 100- or 150-cell blastocyst(21). (ii) There is more uncertainty about impairment on inner cell mass. BB resulted in certain biopsy of extra-embryonic TE cells which have already undergone differentiation, thus leaving the inner cell mass intact. In contrast, each blastomere obtained through CB was considered to have pluripotent property since embryonic genome activation and cell differentiation processes have not yet occurred(22). Consequently, concerns about the degree of impairment on inner cell mass still remain after taking cells with unpredictable developmental fate away during the CB procedure.

No detrimental compromise of CB on implantation potential was observed from initial studies(23). In fact, 2010 ESHRE guidelines suggested that this procedure could be safely applied when embryos are composed of ≥ 6 cells with less than 30% of fragmentation(5). This perception was considered to be absolute until evidence produced by Scott and his colleagues showed otherwise(6). They reported a 39% relative reduction in implantation rate when CB was conducted with respect to self-control but only an absolute reduction of 3% with non-significant difference regarding the BB group versus its paired control(6). This study used an inventive self-paired and randomized design. However, the paired nature of their design dictated that each transfer was only compared with itself but not capable of comparing the impacts of CB versus BB, as indicated by the authors. Besides, the direct implantation clue, observation on the number of viable gestational sacs from ultrasound at early pregnancy was not provided. Hence the question remains unanswered as to when the loss of embryos happened, i.e. implantation failure at the beginning or loss after embedding. If the embryos loss are due to CB occurring before embedding, solutions of helping to alleviate or avoid invasive diagnosis and selecting more competent methods as extended blastocyst culture or simultaneous chromosome screening might be approved. However, if after embedding, the compromise might be to anchor in deficient communication to the endometrium. In addition, very limited sample size was available in this study. In the comparison of cleavage-stage biopsy, only 13 singletons derived from two cleavage-stage embryos transferred could be used to analyze which one was implanted. Among them, 2 singletons were derived from biopsies, and 11 from non-biopsies.

In the first FWBT cycle of the present study, the implantation rate was reduced from 51.8% in BB to 41.0% in CB ($P = 0.044$), which represented a relative reduction of 20.8%. There was a statistically significant difference in the ongoing pregnancy rate between the two groups, with 37.3% in CB and 47.9% in BB ($P = 0.038$). The QBFR post-biopsy was comparable regardless of whether the timing of biopsy was at the cleavage stage or at the blastocyst stage (Group CB: 57.9%; Group BB: 60.5%; $P = 0.139$), which was comparable with previous reports (14, 16). Thus, findings here not only provided direct evidence to corroborate conclusions drawn from the study of Scott *et al.* but also clearly demonstrated that CB might exert adverse impacts mainly on implantation potential after blastocyst formation.

Preliminary data from a previous multi-center study also showed a higher pregnancy rate and implantation rate of 81.3% and 75% for BB with frozen-warmed blastocyst transfer compared to 65.1% and 60% for CB with fresh cleavage-stage embryo transfer cases(24). However, important confounding factors known to affect implantation potential were not adjusted in the analysis. For example, frozen-warmed blastocysts after vitrification were transferred in BB, while fresh cleavage embryos were transferred in CB. When comparisons were conducted between a fresh D3(cleavage-stage)-ET after CB and a deferred blastocyst-ET after BB, the timing of transfer involving fresh or frozen-warmed and transferred embryo stage (involving blastocyst culture or not), should be controlled for. In another study, transferred embryos in both groups were at blastocyst stage, however, bias remained since fresh ET was performed after CB but a frozen-warmed ET was administered after BB. No significant differences in implantation rate (50.3% fresh blastocyst transfer after CB vs. 61.4% FWBT after BB) and clinical pregnancy rate (56.1% vs. 65.3% in two groups respectively) were found between the two groups in the study(17).

Recently, a retrospective study of PGT for sickle cell anemia was implemented to compare outcomes between Day 3 and Day 5/6 biopsy with the same freeze-all strategy and extended blastocyst culture as the present study. The implantation rate and live birth rate were significantly higher in the BB group which presented as 32.1% and 20%, compared with 3.7% and 3.7% in the CB group, respectively(15). However, the CB data were obviously lower than what the majority of papers had reported (7). Since the exact ratio was not provided in that study, it was not known whether less favorable clinical outcomes resulted due to high occurrence of the two-blastomere biopsy process implemented in CB. However, PCR confirmation of CB was merely achieved in 65.7% of cases(15), which was lower than expected on average(25). Another pilot study with a similar methodological comparison as ours tested the implantation rate of PGT for β -thalassemia, and was found to be 47.6% and 26.7% in the BB and CB groups, respectively without statistical differences ($P=0.107$). One limitation was the extremely small sample size, which included 20 sample cycles in total; 10 for each biopsy group(16).

Since prospective randomized controlled trials cannot easily be conducted, PSM was used in the retrospective study as a substitute for randomization. It was performed to take advantage of minimizing selection bias and is partly capable of mimicking RCTs. This procedure was supposed to be a valid statistical strategy for adjustment of imbalances, for the reason that the essential assumption underlying PSM was that subjects with equal propensity scores would be given similar baseline covariate values. Besides, PSM was calculated with the expectation that no unmeasured confounders existed, since the presence of these would account for bias inclusion(26). We primarily used multivariate regression with enter-in methods analysis, and it showed that both endometrium thickness and biopsy methods were significant independent factors. This discrepancy might reduce the correlation of different biopsy methods to clinical outcomes, even though it showed that biopsy strategy was meaningful in pregnancy outcomes. Instead, important confounding factors covering age, basal hormone levels, past miscarriage times (PMT), stimulation protocol, number of oocytes retrieved, and also independent factors associated with first FWBT as the endometrium preparation protocols were set as the PSM independents. Biased confounding variables which were thought to affect PSM, such as endometrium thickness as well as ET-day E_2 levels, were well balanced after matching.

Another advantage of our study was that the CLBR data illuminated the total benefit gained in one OPU-PGT cycle. Through Grey's analysis, distinctive differences were found between groups. The CLBR ranged from 62.1% in the CB group to 80.0% in the BB group in three successive FWBT followed by one OPU cycle, which was a relative gain of 22.4% [$1-(62.1\%/80\%)$].

One limitation of the present study was that no chromosome screening was performed, which may compromise the effectiveness of our results. Indeed, PGT for thalassemia in our center was not compulsory with simultaneous pre-implantation genetic testing for aneuploidies (PGT-A). As such, we were unable to trace whether more aneuploidy blastocysts had been transferred in the CB group, and whether early pregnancy loss could be attributed to the high aneuploidy rate. However, only young females with a mean age less than 35 were included, which implied that there was a relatively low aneuploidy rate in our sample. Next, neither the CB nor BB group had additional PGT-A performed. The

implantation rate in the BB group was quite similar to that calculated in the self-paired study (50%)(6), which could be regarded as tangible evidence of controlled status of independent variables. Besides, cleavage stage for PGT-A has been proved to have less diagnostic reliability due to extremely low DNA content and a high incidence of mosaicism at this stage(27). It has been reported in a systematic review that nearly 30% of cleavage-stage embryos were mosaic and approximately 60% of these diploid-aneuploid mosaics contained euploid cells(28). Therefore, we considered that lack of aneuploidy screening would not adversely influence our results.

Another limitation of our study was the retrospective nature, which may cause unknown bias. Clearly, prospective randomized studies are needed to prove superiority of BB to CB regarding implantation potential and live births. Besides, the number of paired patients after PSM enrollment was limited to 140, which inevitably reduced statistical power. Larger cohorts are needed to confirm our results.

To conclude, biopsy at the blastocyst stage is recommended in favor of CB, as there is a 28.9% increment in implantation rate and a 22.4% increment in cumulative live birth rate.

Declarations

Ethics approval and consent to participate:

Written informed consent was obtained from both couple members. All materials and diagnosis protocols in this study were approved by the Medical Ethical Committee of the First Affiliated Hospital, Sun Yat-sen University, China (Application ID: [2016]160).

Consent for publication:

All the co-authors have reached consensus for publication.

Availability of data and materials:

All the data and materials have been available in the manuscript.

Competing interests:

The authors declare that they have no competing interests.

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Authors' contributions:

Xu Yanwen conceived and designed the study, and revised the manuscript. Wang Yizi designed the study, performed acquisition, analysis and interpretation of data, and drafted the manuscript. Liu Xinyan acquired and analyzed the raw data. Ding Chenhui, Lu Baomin, and Zeng Yanhong arranged and performed specific biopsies, blastocyst culture, embryo freezing and warming cooperatively. Zhou Canquan revised the article. All authors read and approved the final version of the manuscript.

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Tables

Table 1. Primer set and products size for and diagnosis

Primers	Primer sequences (5'-3')	Type	Product size (bp)
S1	GTGTTCTCAGTATTGGAGGGAA		
S2	FAM-GACACGCTTCCAATACGCTTA		Normal (S1+S2):282
S3	HEX-CTACTGCAGCCTTGA ACTCC		
A	GGCCAATCTACTCCCAGGAG		Affected (S1+S3):178
B	ACATCAAGGGTCCCATAGAC		A+B:597
C	ATAACAGTGAATTTCTGG		C+D:362
D	AAAGCGAACTTAGTGATAC		

Table 2. Patient characteristics and Embryology results in CB and BB groups.

Unless otherwise indicated, data were presented as numbers with standard deviation.

Indication	Group CB	Group BB	<i>p</i> -value
Diagnosis	α-thalassemia	β-thalassemia	-
Total patients	202	237	-
Maternal age (years)	30.93.3(22-34)	31.25.6(21-34)	0.525
BMI, kg/m ²	21.42.3	20.52.1	0.214
Basic FSH (IU/L)	5.71.4(3.21-9.16)	5.93.6(3.76-9.79)	0.367
Basic LH (IU/L)	4.02.1(1.49-9.71)	3.81.8(1.89-8.81)	0.359
Basic E ₂ (pg/μl)	32.510.6(19-74)	33.715.4(10-87)	0.347
Miscarriage times; n			
0	80	129	
1	92	75	
≥2	30	33	0.005
Day of Biopsy	D3	D5, D6	-
Biopsy procedure			
Zona breaching	Laser	Laser	-
Biopsy method	Blastomere	Trophectoderm	-
Stage of embryo transferred	Blastocyst (Day 5 or 6)	Blastocyst (Day 5 or 6)	-
Type of transferred embryo	Thaw	Thaw	-
Cryopreservation method	Vitrification	Vitrification	-
Embryology ^a			
QCSEs ^b	9 (3-19)	8 (4-16)	-
Qualified blastocysts ^c	5 (2-12)	5 (3-11)	-
QBFR (%) ^d	57.9	60.5	0.139
Total qualified blastocysts; n	1089	1205	-
Embryo genotype; n			
Diagnosed (%)	1005 (92.3)	1126 (93.4)	0.281
Transferrable blastocysts ^e per cycle; n	3.04±1.95	3.4±1.32	0.152

^aData are positively skewed by distribution, presented as median number with ranges in parentheses.

^bQCSEs: qualified cleavage-stage embryos; embryos that had at least five or more cells with <50% of fragmentation early on day 3 after oocyte retrieval.

^c Qualified blastocysts: blastocysts graded better than 3BC/3CB.

^dQBFR: qualified blastocyst formation rate, calculated by Qualified blastocysts/QCSEs.

^etransferrable blastocysts referred to blastocysts diagnosed as normo-homozygote or heterozygote genotype.

Differences were statistically significant as $p < 0.05$.

Table 3. Cycle Characteristics from the First Frozen-thaw Embryo Transfer before PSM

	Group CB	Group BB	<i>p</i> -value
Survival rate after warming (%)	97.7%	98.1%	-
Embryo transfer cycle; n	202	237	-
Protocol; n			
Natural	83	97	0.973
HRT	119	140	
Transferred blastocysts grade; n			
excellent	101	115	
good	136	148	0.125
Average	45	76	
Average number transferred ^a	1.400.49	1.280.45	0.867
Total embryo transferred; n	282	339	-
One-blastocyst transfer cycle n (%)	122(43.3)	145(42.8)	
Two-blastocyst transfer cycle n (%)	80(56.7)	92(57.2)	
Endometrial thickness on ET day ^a	9.63.8	11.42.7	0.037
Time interval ^c (days)	81.119.3	94.25.1	0.003
E ₂ on ET day(pg/ml) ^b	125 (62.9-2580)	91.3 (43-2300)	0.019
P ₂ on ET day (ng/ml) ^a	11.23.7	10.62.9	0.519
Luteal Support protocol; n (%)			
Univocal	30 (14.9)	47 (19.8)	
Compound	172 (85.1)	190 (80.2)	0.208

^a Data were presented as numbers with standard deviation.

^bData positively skewed by distribution, presented as median number with ranges in parentheses.

^c Time Interval—the last ovum pick-up (OPU) day of stimulation cycle and embryo transfer day of subsequent first FWBT cycle.

ET: embryo transfer. PSM: propensity score matching.

Differences were statistically significant as $p < 0.05$.

Table 4. Selected matching variables in first FET cycle of study patients before and after PSM analysis

Before PSM				After PSM		
Variable	Group CB (n = 202)	Group BB (n = 237)	<i>p</i> -value	Group CB (n = 140)	Group BB (n = 140)	<i>p</i> -value
PMT ^a						
0	80	129	0.005	84	82	0.813
1	92	75		46	50	
≥2	30	33		10	8	
Endometrial thickness ^b						
	9.63.8	11.44.7	0.036	9.72.9	9.93.2	0.741
E ₂ ^c (pg/ml)	125(62.9-2580)	91.3(43-2300)	0.019	102(72-2213)	97(58.2-2140)	0.502

^aPMT: Past miscarriage times.

^bthickness on embryo transfer day.

^cData positively skewed by distribution, presented as median number with ranges in parentheses.

Differences were statistically significant as $p < 0.05$.

Table 5. Cumulative cycle characteristics of FTBT in paired groups after PSM

	Group CB	Group BB	<i>p</i> -value
Patients; n	140	140	-
Total Cycle; n	275	250	-
Maternal age (years)	29.82.6	30.63.2	0.318
Protocol; n (%)			
Natural	86 (31.3)	67 (26.8)	0.260
HRT	189 (68.7)	183 (73.2)	
Transferred blastocysts grade; n			
excellent	111	100	
good	181	172	0.102
Average	76	46	
Total embryo transferred; n	368	318	-
One-blastocyst transfer cycle;	182 (66.2)	183 (73.2)	
n (%)			0.081
Two-blastocyst transfer cycle;	93 (33.8)	67 (26.8)	
n (%)			

Differences were statistically significant as $p < 0.05$.

Table 6. Total reproductive outcome of FTBT cycles

FTBT	Reproductive outcome	Group CB	Group BB	Chi-Square	p-value
Cycle				value	
1st	Cycles; n	140	140		
	Embryos transferred; n	178	170		
	Positive β -HCG(≥ 10 IU/L); n (%)	82 (57.7)	89 (62.7)	0.736	0.391
	Implantation Embryos; n (%) ^a	73 (41.0)	88 (51.8)	4.045	0.044
	Ongoing pregnancy; n (%) ^b	53 (37.3)	70 (47.9)	4.415	0.042
	Live birth per FET cycle with embryo transfer; n (%)	56 (31.4)	73 (42.9)	4.913	0.027
	Clinical miscarriage rate	11.6	7.7		0.032
2nd	Cycles; n	88	72		
	Embryos transferred; n	119	92		
	Positive β -HCG(≥ 10 IU/L); n (%)	43 (48.8)	39 (54.2)	0.446	0.504
	Implantation Embryos; n (%) ^a	36 (30.3)	40 (43.5)	3.939	0.047
	Ongoing pregnancy; n (%) ^b	22 (25.0)	27 (37.5)	2.912	0.088
	Live birth per FET cycle with embryo transfer; n (%)	24 (20.2)	30 (32.6)	4.217	0.040
	Cumulative Live birth; n (%)	80/140 (57.1)	103/140 (73.6)		
	Clinical miscarriage rate	9.6	8.5		0.681
3rd	Cycles; n	35	25		
	Embryos transferred; n	54	42		
	Positive β -HCG(≥ 10 IU/L); n (%)	17 (48.5)	14 (48)	0.322	0.570
	Implantation Embryos; n (%) ^a	11 (20.4)	17 (40.5)	4.257	0.039
	Ongoing pregnancy; n (%) ^b	6 (17.1)	4 (32)	1.799	0.180
	Live birth per FET cycle with embryo transfer; n (%)	7 (13.0)	9 (21.4)	1.219	0.270
	Clinical miscarriage rate	10.3	8.9		0.078
	Cumulative Live birth; n (%)	87/140 (62.1)	112/140 (80.0)	-	0.032 [#]

^aImplantation rate: the number of detected gestational sacs detected by transvaginal ultrasound at 6-8 weeks compared with the number of total embryos transferred.

^bOngoing pregnancy rate: the cycles of neonatal birth until 28 gestational weeks or more compared with corresponding total transferred cycles.

^cMiscarriage rate: Cycles of spontaneous pregnancy loss (excluded biochemical pregnancy and multiple pregnancy reduction)/successful implantation cycle

[#]Value calculated by Fine-Gray test in presence of competing risks

Differences were statistically significant as $p < 0.05$.

Table 7. Fine-Gray regression analysis of live birth rate by using competing risk model in the first FWBT of initial group.

Group	Fine-Gray regression analysis		
	Coefficient	HR (95%CI)	<i>p</i> -value
Biopsy strategy			
CB	-	-	-
BB	0.82	1.21 (1.06-4.35)	0.041
Endometrial Thickness			
<5mm	-	-	-
5-8mm	0.85	2.31 (1.39-4.47)	0.005
>8mm	0.91	3.52 (2.02-5.11)	<0.001

HR: Hazard ratio. Differences were statistically significant as $p < 0.05$.

Figures

Figure 1

(a) Cumulative live birth rate (CLBR) of three consecutive frozen-warmed blastocyst transfer (FWBT) cycles after one oocyte retrieval procedure. The CLBR was 62.1% in the Cleavage-stage Biopsy (CB) Group, significantly lower than 80.0% of Blastocyst-stage biopsy (BB) Group (Fig1, 2) in 140 cycles after propensity score matching. (b) Grey's competing risk analysis of CLBR in the two groups. A statistically significant increment of 17.9% CLBR gained in Group BB compared to CB ($P = 0.032$). (Red line: Group CB, Blue line: Group BB).