Transfer of hatched blastocyst can result in skewed sex ratio:
a retrospective cohort study

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Research

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Abstract

Background

The correlation between blastocyst quality and birthweight, neonatal outcomes is still controversial. There is a significantly higher male: female ratio among good quality blastocysts (advanced trophoderm morphology) but in the expansion degree, the significance for sex ratio is unclear.

Methods

A total of 617 and 6803 live singleton births resulting from the transfer of fresh and frozen-thawed single blastocysts in the Reproductive Medicine Center of Peking University Third Hospital from 2009 to 2020 were included. Live singleton births from fresh and frozen-thawed single blastocyst transfer were stratified by inner cell mass/trophoderm morphology and degree of blastocoel expansion. Multivariate linear regression was used to analyze the correlation between expansion, inner cell mass/trophoderm morphology, and birthweight, Z score, gestational weeks. Logistic regression was used to analyze the relationship between expansion, ICM/TE morphology and sex, neonatal outcomes.

Results

There was no significant correlation between birthweight, neonatal outcomes and blastocyst quality in fresh and frozen-thawed single blastocyst transfer cycles. However, the proportion of male infants in the hatched blastocyst (stage-6) group (67.9% vs. 54.2%; p < 0.001) [OR: 1.76 95% CI (1.34–2.32)] and hatching blastocyst (stage-5) group (61.7% vs. 54.2%; p = 0.001) [OR: 1.36 95% C.I (1.14–1.62)] was significantly higher than that in the expanded blastocyst (stage-4) group.

Conclusions

The transfer of poor-quality blastocysts is unlikely to affect birthweight and neonatal health; however, transfer of stage-6 blastocysts can result in extremely skewed sex ratio.

Background

Embryo quality is the key factor affecting the success rate of assisted reproductive technology (ART). The degree of blastocoel expansion and the inner cell mass (ICM)/trophoderm (TE) morphology are often used to evaluate blastocyst quality based on Gardner criteria [1]. According to the size of the blastocyst cavity and the signs of hatching or not, blastocyst development was divided into six stages. In stage 3–6 blastocysts, the ICM/TE morphology is assessed from grade A to C. Several studies have reported that there is a strong correlation between blastocyst quality, developmental stage (day 5 vs. day 6), and live birth rate [2-8].

The ultimate goal of ART is to transfer an embryo and to give birth to a healthy baby [9]. Compared with natural pregnancies, singletons born by ART have higher risks of preterm birth (PTB), low birthweight (LBW), fetal mortality, and birth defects [10-13]. Furthermore, another study has reported that the risk of pregnancy complications is significantly increased in women who have undergone ART [13]. The etiology of infertility may be the main cause of adverse obstetric and neonatal outcomes, but ART also plays a role [12]. Research has shown that the risk of PTB is significantly higher in cases of blastocyst transfer than that in cases of cleavage-stage embryo transfer [14, 15]. Compared with fresh embryo transfer, the risks of pre eclampsia and large for gestational age (LGA) are increased in frozen embryo transfer [16-18].

However, the relationship between blastocyst quality and IVF neonatal outcomes is still controversial. In a few studies, there was no significant correlation between blastocyst quality and neonatal outcomes, such as LBW, small for gestational age
(SGA), and LGA [4, 19, 20]. However, another study has reported that the transfer of poor quality blastocysts (3-6BC, CB, CC) significantly reduced birthweight compared with the transfer of high quality blastocysts (3-6AA) [21]. The subjective morphological evaluation and different embryo transfer standards (grade CC embryos are usually discarded) may be the main reasons for the inconsistency across results. Therefore, additional studies are needed to further confirm the relationship between blastocyst quality, developmental stage, and neonatal outcomes.

We retrospectively analyzed 617 live singleton births born from the transfer of fresh single embryos and 6803 live singleton births born from the transfer of frozen-thawed single embryos to investigate the independent effects of blastocyst quality, including degree of blastocoel expansion, and developmental stage on neonatal outcomes. Based on our previous findings with the transfer of cleavage-stage embryos [22], it was hypothesized that blastocyst quality is unlikely to cause adverse neonatal outcomes; however, blastocyst expansion is likely to cause bias of sex ratio after adjusting for confounder with multivariate regression analysis.

**Methods**

**Participants**

This study retrospectively analyzed 617 and 6803 live singleton births resulting from the transfer of fresh or frozen-thawed single blastocysts in the Reproductive Medicine Center of Peking University Third Hospital from 2009 to 2020. Pregnancy complications that significantly affected birthweight (gestational diabetes, gestational hypertension, pre-eclampsia), monozygotic twin births, and a history of pre-implantation cycles were excluded. For the transfer of fresh blastocysts, good quality blastocysts were defined as ≥3-6BB, poor quality blastocysts were defined as 3-6BC/CB, and early blastocysts (stage 1 or 2) [5]. There were 494 singletons in the good quality blastocyst group and 123 singletons in the poor quality blastocyst group. There were 506 singletons in the day-5 blastocyst transfer group and 111 singletons in the day-6 blastocyst transfer group. Early and full blastocysts were defined as non-expanded blastocysts, and there were 112 singletons in the non-expanded blastocyst group. Expanded, hatching, and hatched blastocysts were defined as expanded blastocysts, and there were 505 singletons in the expanded blastocyst group. For the transfer of frozen-thawed blastocysts, high quality blastocysts were defined as 3-6AA, and there were 373 singletons in this group. Good quality blastocysts were defined as 3-6AB or BA, and there were 1200 singletons in this group. Fair quality blastocysts were defined as 3-6BB, and there were 3495 singletons in this group. Poor quality blastocysts were defined as 3-6BC or CB, and there were 1735 singletons in this group. For both fresh and frozen-thawed cycles, ICM/TE morphology with grade C was defined as poor quality.

**Laboratory protocol**

IVF and ICSI were performed according to routine laboratory insemination procedures on the day of oocyte retrieval. The presence of two pronuclei was observed 17–19 h after insemination or injection, and the zygotes were then cultured in 25-ml droplets of pre-equilibrated G1-Plus medium (Vitrolife, Gothenburg, Sweden). The morphology of embryos was evaluated with respect to cell number, fragmentation, and symmetry 68–72 h after insemination. Generally, high quality embryos were transferred on day 3 or frozen at this stage. The remaining good quality embryos were placed in G2-plus medium (Vitrolife) until they reached the blastocyst stage. Blastocysts that reached the fully expanded, hatching, or hatched stage and earned a score above grade 4CC according to Gardner criteria were transferred or cryopreserved. In some cases, early blastocysts were also transferred in fresh cycles. From the beginning of 2019, day-6 blastocysts were not transferred in fresh cycles and cryopreserved for subsequent thawed cycles in our center.

The expanded blastocysts collapsed after artificial shrinkage, which was followed by vitrification and warming. Briefly, the blastocysts were equilibrated in 7.5% (v/v) dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO, USA) and 7.5% (v/v) ethylene glycol (EG; Sigma Chemical Co.) at 37°C for 2 min and placed in 15% DMSO, 15% EG, and 0.65 mol/l sucrose for 30 s. During this period, one blastocyst was placed on the Cryotop strip (Kitazato, Fuji, Japan), which was then
immediately plunged into liquid nitrogen. For warming, the Cryotop was quickly placed in 0.33 mol/l sucrose at 37°C. After 2 min, the blastocysts were transferred to 0.2 mol/l sucrose for 3 min, followed by HEPES-buffered medium for 5 min. Thereafter, the blastocysts were cultured in G2-Plus medium for 2 h to evaluate their quality. Blastocysts with good survival (less than one-half of the blastocysts showing signs of damage) and re-expansion were transferred. DMSO, EG, and sucrose, as cryoprotectants, were also used for day-3 embryo freezing and warming.

Assessment of blastocyst morphology

According to the size of the blastocyst cavity and the signs of hatching or not, blastocyst development was divided into six stages. Stage 1 was defined as early blastocysts with a cavity, with the volume of the blastocyst cavity being less than 1/2 of the total embryo volume. Stage 2 was defined as blastocysts with a cavity that was greater than or equal to 1/2 of the embryo volume. Stage 3 was defined as blastocysts with a cavity that completely occupied the total volume of the embryo. Stage 4 was defined as expanded blastocysts, with the cavity completely occupying the embryo, with the total volume of the embryo becoming larger and the zona pellucida (ZP) becoming thinner. Stage 5 was defined as hatching blastocysts, with a portion of the blastocyst escaping from the ZP. Stage 6 was defined as hatched blastocysts, with the blastocysts completely escaping from the ZP. In stage 3–6 blastocysts, the development of the ICM was assessed as follows: A indicated many tightly packed cells; B indicated many loosely grouped cells; and C indicated very few cells. The TE was assessed as follows: A indicated many cells forming a cohesive epithelium; B indicated few cells forming a loose epithelium; and C indicated very few large cells.

Outcomes measured

Neonatal outcomes were evaluated as follows: LBW (<2500 g), PTB (<37 weeks), SGA, and LGA. The SGA cohort represented the newborns with a birthweight below the lower 10th percentile for gestational age. The LGA cohort represented the newborns with a birthweight above the upper 10th percentile for gestational age. Birthweight data were acquired by telephone interview within 1 week of birth. To calculate the proportions of SGA and LGA, we used a previous publication that provided Chinese birthweight references at different gestational ages, including standard deviations [23]. We also calculated gestational age- and gender-adjusted birthweights (also known as the z score) for all IVF babies using the equation \( Z = \frac{x - \mu}{s} \), where \( x \) is the IVF birthweight, \( \mu \) is the mean birthweight at the same gestational age and same gender in the reference group and, \( s \) is the standard deviation in the respective reference group.

Statistical analysis

Statistical analysis was performed using Statistical Package for Social Science (SPSS) software, version 25.0 (IBM, Armonk, New York, USA). Student’s t test and one-way analysis of variance (ANOVA) were used to compare measurement data, as appropriate. Comparisons between categorical data were performed using the chi-square test. Multiple linear regression analyses were used to evaluate possible correlations between birthweight, z score, gestational age, and blastocyst quality, while adjusting for other confounding factors, including developmental stage, degree of expansion, female age, female BMI, parity, insemination method [in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI)], number of oocytes retrieved, gender, and gestational age. Binary logistic regression was used to evaluate possible correlations between blastocyst quality and neonatal outcomes, while adjusting for other confounding factors, including developmental stage, degree of blastocoel expansion, female age, female BMI, parity, insemination method, and number of oocytes retrieved. All reported P-values were two tailed, and \( P < 0.05 \) was established as the level of significance.

Results

A total of 617 live singleton births born from fresh cycles and 6803 live singleton births born from frozen-thawed cycles were included in this study. The basic characteristics of patients and cycles are presented in Tables 1 and 2. The average age of women was 32.4 ± 4.1 years in the fresh cycle group and 31.9 ± 4.0 years in the frozen-thawed cycle group. Primipara accounted for 84.3%, with multipara accounting for 15.7% of fresh cycle cases. Primipara accounted for 93.9%, with
multipara accounting for 6.1% of frozen-thawed cycle cases. In the fresh cycle group, 82.0% and 18.0% of infants were born with blastocysts transferred on day 5 and day 6; 18.2% and 81.8% of infants were born with stage 1, 2, 3 and 4, 5, 6 blastocysts; and 80.1% and 19.9% of infants were born with good quality and poor quality blastocysts, respectively. In the frozen-thawed cycle group, 2.3%, 85.7%, 8.5%, and 3.6% of infants were born with stage 3, 4, 5, and 6 blastocysts; and 5.5%, 17.6%, 51.4%, and 25.5% of infants were born with high, good, fair, and poor quality blastocysts, respectively.

Table 1
Patient and cycle characteristics in fresh cycles

|                        | Singleton (n=617) |
|------------------------|------------------|
| Female age             | 32.4±4.1         |
| Female BMI             | 22.9±3.6         |
| Oocyte number          | 13.1±6.2         |
| parity                 |                  |
| Primipara              | 520 (84.3%)      |
| multipara              | 97 (15.7%)       |
| Insemination method    |                  |
| IVF                    | 398 (64.5%)      |
| ICSI                   | 219 (35.5%)      |
| Developmental Stage    |                  |
| Day 5                  | 506 (82.0%)      |
| Day 6                  | 111 (18.0%)      |
| Expansion              |                  |
| Early and full blastocyst (1a,2b,3 stage) | 112 (18.2%) |
| Expanded blastocyst (4,5c,6d stage) | 505 (81.8%) |
| Morphology             |                  |
| Good quality           | 494 (80.1%)      |
| Poor quality           | 123 (19.9%)      |

Data are presented as numbers (%) or mean±SD. a,b Because the sample size of stage-1 (n = 13) and -2 (n = 20) blastocysts was very small, they were combined with stage-3 blastocysts in the subsequent multivariate regression analysis. c,d Because the sample size of stage-5 (n = 13) and -6 (n = 7) blastocysts was very small, they were combined with stage-4 blastocysts in the subsequent multivariate regression analysis.
Table 2
Patient and cycle characteristics in frozen-thawed cycles

|                        | Singleton (n=6803) |
|------------------------|-------------------|
| **Female age**         | 31.9±4.0          |
| **parity**             |                   |
| Primipara              | 6387 (93.9%)      |
| multipara              | 416 (6.1%)        |
| **Endometrial preparation** |               |
| Artificial cycle       | 3015 (44.3%)      |
| Natural cycle          | 3788 (55.7%)      |
| **Degree of expansion**|                   |
| 3                      | 150 (2.2%)        |
| 4                      | 5828 (85.7%)      |
| 5                      | 579 (8.5%)        |
| 6                      | 246 (3.6%)        |
| **Morphology**         |                   |
| High                   | 373 (5.5%)        |
| Good                   | 1200 (17.6%)      |
| Fair                   | 3495 (51.4%)      |
| Poor                   | 1735 (25.5%)      |

Data are presented as numbers (%) or mean+SD.

Table 3 shows birthweight, gestational week, neonatal outcomes of blastocyst quality, degree of blastocoel expansion, and developmental stage for the fresh cycle group. The proportion of males in the good quality blastocyst group was significantly higher than that in the poor quality blastocyst group (61.5% vs. 47.2%; p = 0.004). Moreover, the proportion of newborns with LGA in the expanded, hatching, and hatched blastocyst group was significantly higher than that in the early and full blastocyst group (18.4% vs. 8.9%; p = 0.015). Other parameters were not significantly different between good and poor quality blastocyst groups, expanded and non-expanded groups, and day-5 and day-6 blastocyst groups.
Table 3  
Blastocyst quality, degree of expansion, developmental stage, and neonatal outcomes in fresh cycles

|                        | good quality blastocyst (n=494) | poor quality blastocyst (n=123) | p value | early and full blastocyst (n=112) | p value | expanded, hatching, hatched blastocyst (n=505) | p value | day 5 blastocyst (n=506) | p value | day 6 blastocyst (n=111) | p value |
|------------------------|----------------------------------|----------------------------------|---------|-----------------------------------|---------|-----------------------------------------------|---------|------------------------|---------|------------------------|---------|
| Gestational Age        | 38.3±1.6                         | 38.3±1.9                         | 0.707   | 38.3±2.1                          | 0.964   | 38.2±1.7                                      | 0.964   | 38.4±1.6               | 0.371   |
| Birthweight            | 3328±510                         | 3268±520                         | 0.245   | 3244±517                          | 0.099   | 3306±510                                      | 0.261   | 3366±522               | 0.852   |
| Gender (male percent)  | 304 (61.5%)                      | 58 (47.2%)                       | 0.004   | 62 (55.4%)                        | 0.431   | 296 (58.5%)                                   | 0.852   | 66 (59.5%)            | 0.916   |
| PTB                    | 43 (8.7%)                        | 11 (8.9%)                        | 0.933   | 12 (10.7%)                        | 0.417   | 44 (8.7%)                                     | 0.916   | 10 (9.0%)            | 0.839   |
| LBW                    | 23 (4.7%)                        | 8 (6.5%)                         | 0.401   | 7 (6.3%)                          | 0.512   | 25 (4.9%)                                     | 0.839   | 6 (5.4%)             | 0.387   |
| SGA                    | 27 (5.5%)                        | 7 (5.7%)                         | 0.922   | 5 (4.5%)                          | 0.592   | 26 (5.1%)                                     | 0.387   | 8 (7.2%)             | 0.329   |
| LGA                    | 88 (17.8%)                       | 15 (12.2%)                       | 0.135   | 10 (8.9%)                         | 0.015   | 81 (16.0%)                                    | 0.329   | 22 (19.8%)          | 0.329   |

Data are presented as numbers (%) or mean±SD. Continuous variables were compared using Student's t test, and categorical variables were evaluated with Pearson's Chi-square tests.

Table 4 shows birthweight, gestational week, neonatal outcomes of blastocyst quality, and degree of blastocoel expansion for the frozen-thawed cycle group. The proportion of males in the high quality blastocyst group (59.8% vs. 51.1%; p < 0.001) and the stage-6 blastocyst group (67.9% vs. 54.2%; p < 0.001) was significantly higher than that in the poor quality blastocyst group and the stage-4 blastocyst group. Other parameters were not significantly different, after stratifying by blastocyst quality and degree of blastocoel expansion.
Table 4
Blastocyst quality, degree of expansion, and birthweight, neonatal outcomes in frozen-thawed cycles

|                              | Group 1 | Group 2 | Group 3 | Group 4 | p value |
|------------------------------|---------|---------|---------|---------|---------|
| **Blastocyst quality**       |         |         |         |         |         |
| High (AA, n=373)             |         |         |         |         |         |
| Good (AB,BA: n=1200)         |         |         |         |         |         |
| Fair (BB, n=3495)            |         |         |         |         |         |
| Poor (BC,CB, n=1735)         |         |         |         |         |         |
| **Birthweight (g)**          | 3386±429| 3377±501| 3398±495| 3386±486| 0.613   |
| **gestational age (weeks)**  | 38.4±1.3| 38.5±1.6| 38.4±1.6| 38.4±1.6| 0.781   |
| **Gender (male percent)**    | 223 (59.8%) | 673 (56.1%) | 1989 (56.9%) | 887 (51.1%) | <0.001 |
| PTB                          | 29 (7.8%) | 96 (8.0%) | 274 (7.8%) | 140 (8.1%) | 0.991   |
| LBW                          | 9 (2.4%)  | 48 (4.0%) | 113 (3.2%) | 60 (3.5%)  | 0.433   |
| SGA                          | 9 (2.4%)  | 45 (3.8%) | 121 (3.5%) | 78 (4.5%)  | 0.148   |
| LGA                          | 68 (18.2%) | 236 (19.7%) | 763 (21.8%) | 375 (21.6%) | 0.198   |
| **Degree of expansion**      |         |         |         |         |         |
| Stage 3 (n = 150)            |         |         |         |         |         |
| Stage 4 (n = 5828)           |         |         |         |         |         |
| Stage 5 (n = 579)            |         |         |         |         |         |
| Stage 6 (n = 246)            |         |         |         |         |         |
| **Birthweight (g)**          | 3336±526 | 3389±483 | 3399±529 | 3434±532 | 0.259   |
| **gestational age (weeks)**  | 38.3±1.7 | 38.5±1.6 | 38.5±1.6 | 38.3±1.6 | 0.310   |
| **Gender (male percent)**    | 89 (59.3%) | 3159 (54.2%) | 357 (61.7%) | 167 (67.9%) | <0.001 |
| PTB                          | 15 (10.0%) | 452 (7.8%) | 48 (8.3%) | 24 (9.8%) | 0.503   |
| LBW                          | 5 (3.3%)  | 193 (3.3%) | 24 (4.2%) | 8 (3.3%)  | 0.769   |
| SGA                          | 7 (4.7%)  | 217 (3.7%) | 24 (4.2%) | 5 (2.0%)  | 0.453   |
| LGA                          | 28 (18.7%) | 1224 (21.0%) | 129 (22.3%) | 61 (24.8%) | 0.388   |

Data are presented as numbers (%) or mean±SD. Continuous variables were compared using one-way ANOVA, and categorical variables were evaluated with Pearson's Chi-square tests.

The results of multivariate linear regression analysis are shown in Table 5. In the fresh cycle group, blastocyst quality, degree of blastocoel expansion, and developmental stage had no significant correlation with birthweight, z score, and gestational week. In the frozen-thawed cycle group, there was no significant correlation between blastocyst quality, degree of blastocoel expansion and birthweight, z score, gestational week.
### Table 5
Linear regression analysis of correlations between blastocyst quality, developmental stage, degree of expansion, birthweight, z score, and gestational age

|                        | birthweight |          |          | Z score |          |          | gestational |          |
|------------------------|-------------|----------|----------|---------|----------|----------|-------------|----------|
|                        | β           | 95% C.I  | p value  | β       | 95% C.I  | p value  | β           | 95% C.I  | p value |
| Fresh cycles\(^a\)    |             |          |          |         |          |          |             |          |         |
| Blastocyst stage       |             |          |          |         |          |          |             |          |         |
| (day6 vs. day5)        | 25.10       | -61.82-112.03 | 0.571 | 0.13 | -0.09-0.35 | 0.249 | 0.24 | -0.11-0.60 | 0.178 |
| Blastocyst Expansion   |             |          |          |         |          |          |             |          |         |
| (4, 5and 6 vs.1,2,and 3) | 55.96      | -31.59-143.51 | 0.210 | 0.09 | -0.14-0.31 | 0.460 | 0.04 | -0.32-0.40 | 0.829 |
| Blastocyst quality     |             |          |          |         |          |          |             |          |         |
| (poor vs. good)        | -19.05      | -104.36-62.26 | 0.661 | -0.09 | -0.31-0.13 | 0.415 | 0.06 | -0.28-0.41 | 0.715 |
| Frozen-thawed cycles\(^b\) |           |          |          |         |          |          |             |          |         |
| Stage 3 (ref)          |             |          |          |         |          |          |             |          |         |
| Stage 4                | -7.85       | -36.68-20.98 | 0.594 | -0.02 | -0.10-0.05 | 0.518 | 0.06 | -0.05-0.16 | 0.300 |
| Stage 5                | -1.94       | -38.12-34.25 | 0.917 | 0.00 | -0.09-0.09 | 0.995 | 0.01 | -0.13-0.14 | 0.912 |
| Stage 6                | 49.82       | -4.32-103.95 | 0.071 | 0.13 | -0.004-0.27 | 0.056 | -0.10 | -0.30-0.10 | 0.316 |
| High quality (ref)     |             |          |          |         |          |          |             |          |         |
| Good                   | -18.84      | -45.33-7.75 | 0.163 | -0.03 | -0.10-0.03 | 0.329 | 0.02 | -0.08-0.12 | 0.721 |
| fair                   | 8.38        | -11.82-28.58 | 0.416 | 0.02 | -0.03-0.08 | 0.351 | 0.01 | -0.06-0.09 | 0.713 |
| poor                   | 4.71        | -18.44-27.86 | 0.690 | 0.004 | -0.06-0.06 | 0.883 | -0.02 | -0.10-0.07 | 0.662 |

\(^a\) adjusted by female age, female BMI, parity, number of oocytes, and insemination method

\(^b\) adjusted by female age, parity, and endometrial preparation

The results of multivariate logistic regression analyses are shown in Table 6. In the fresh cycle group, blastocyst quality, degree of blastocoel expansion, and developmental stage had no significant correlation with neonatal outcomes. In the frozen-thawed cycle group, there was no significant correlation between blastocyst quality, degree of blastocoel expansion, and neonatal outcomes. However, stage-5 [OR: 1.36, 95% C.I (1.14–1.62)] and stage-6 [OR: 1.76 95% C.I (1.34–2.32)] blastocyst groups had a significantly higher proportion of males than the stage-4 blastocyst group.
Table 6
Logistic regression analysis of correlations between blastocyst quality, developmental stage, degree of expansion, and neonatal outcomes in fresh cycles

|                        | PTB   | LBW   | SGA   | LGA   | Gender |
|------------------------|-------|-------|-------|-------|--------|
| **Fresh cycles**       |       |       |       |       |        |
| Blastocyst quality     | 0.94  | 1.30  | 1.04  | 0.81  | 0.52   |
| (ref: good)            | (0.45,1.94) | (0.54,3.11) | (0.43,2.53) | (0.43,1.50) | (0.34,0.80) |
| Developmental stage    | 1.04  | 0.96  | 1.34  | 1.33  | 1.09   |
| (ref: day5)            | (0.49,2.22) | (0.37,2.49) | (0.57,3.14) | (0.76,2.31) | (0.70,1.70) |
| Degree of expansion    | 0.73  | 0.79  | 1.31  | 2.05  | 0.99   |
| (stage1,2,3)           | (0.36,1.50) | (0.32,1.97) | (0.48,3.57) | (1.00,4.21) | (0.64,1.54) |
| **Frozen-thawed cycles** |       |       |       |       |        |
| High quality (ref)     |       |       |       |       | 1.21   |
| Good quality           | 1.03  | 1.69  | 1.58  | 1.12  | 0.86   |
| (0.67,1.60)            | (0.82,3.49) | (0.77,3.27) | (0.83,1.51) | (0.67,1.09) |        |
| Fair quality           | 1.00  | 1.35  | 1.47  | 1.25  | 0.87   |
| (0.67,1.49)            | (0.68,2.70) | (0.74,2.92) | (0.95,1.66) | (0.70,1.08) |        |
| Poor quality           | 1.06  | 1.47  | 1.93  | 1.25  | 0.70   |
| (0.70,1.61)            | (0.72,3.00) | (0.96,3.88) | (0.94,1.67) | (0.56,0.88) |        |
| Stage 3 (ref)          |       |       |       |       | 1.21   |
| stage 4                | 0.72  | 0.91  | 0.72  | 1.13  | ref    |
| (0.42,1.24)            | (0.37,2.26) | (0.33,1.57) | (0.74,1.71) |        |        |
| stage 5                | 0.79  | 1.17  | 0.82  | 1.22  | 1.36   |
| (0.43,1.45)            | (0.44,3.12) | (0.35,1.96) | (0.77,1.93) | (1.14,1.62) |        |
| stage 6                | 0.95  | 0.91  | 0.40  | 1.39  | 1.76   |
| (0.48,1.87)            | (0.29,2.85) | (0.12,1.29) | (0.84,2.31) | (1.34,2.32) |        |

Data are presented as adjusted odd risk (95% CI)

- **a** adjusted by developmental stage, degree of expansion, female age, female BMI, parity, insemination method, and oocyte number
- **b** adjusted by blastocyst quality, degree of expansion, female age, female BMI, parity, insemination method, and oocyte number
- **c** adjusted by developmental stage, blastocyst quality, female age, female BMI, parity, insemination method, and oocyte number
- **d** adjusted by female age, parity, endometrial preparation, and degree of expansion
- **e** adjusted by female age, parity, endometrial preparation, and blastocyst quality
- **f** reference group is stage-4 blastocysts

**Discussion**

This study found that blastocyst quality, degree of blastocoel expansion, and developmental stage were not significantly correlated with birthweight, gestational week, z score, and neonatal outcomes, including, PTB, LBW, SGA, and LGA. However,
blastocysts with an advanced degree of blastocoel expansion were likely to result in a significantly higher proportion of male infants.

Earlier studies have shown that the transfer of poor quality cleavage-stage embryos or blastocysts did not increase the risk of adverse neonatal outcomes, and there was no significant difference in birthweight between the two groups [19, 22]. In a previous study, the sample size of poor quality blastocysts was very small and did not provide reliable evidence [19]. Subsequently, the research team suggested that almost all obstetric and neonatal outcomes had no significant differences in the four groups of high, average, and low quality, as well as stage-1/2 early blastocysts [20]. In this study, after adjusting for confounding factors, the sex ratio of newborns in average and low quality, as well as early blastocysts, was significantly lower than that in high quality blastocysts [20]. Consistent with this finding in our study, there was a lower proportion of male infants in the poor quality blastocyst group in both fresh and frozen-thawed cycles. However, to our knowledge, this is the first study to suggest that the proportion of male infants increased with advanced blastocoel expansion, and the proportion of male infants in stage-5 and -6 blastocyst groups was significantly higher than that in the stage-4 blastocyst group.

A recent study showed that the birthweight of newborns born with poor quality embryos (3-6BC, CB, CC) decreased significantly compared with high quality embryos (3-6AA), and the high quality blastocyst group had a higher incidence of LGA [21]. This study used the ICM/TE morphology as an indicator of blastocyst quality, but it did not include the degree of blastocoel expansion and the developmental stage. In previous studies, the developmental stage was reported to be significantly correlated with birthweight, PTB, and LGA [14, 15, 24-26]. Thus, the developmental stage may be an important factor that affects adverse neonatal outcomes. The degree of blastocoel expansion was significantly correlated with the success rate of ART. The probability of live birth with expanded and hatching blastocysts was significantly higher than that of early and fully expanded blastocysts [5]. The re-expansion rate of frozen-thawed blastocysts was significantly positively correlated with the clinical pregnancy rate [27]. However, there are few reports on the relationship between the degree of blastocoel expansion and neonatal outcomes. To our knowledge, only two articles have reported that the degree of blastocyst expansion had no significant correlation with birthweight and neonatal outcomes [4, 5]. Given the contradiction between blastocyst quality and birthweight, neonatal outcomes, our study, which included the largest sample size of newborns, evaluated the effects of all blastocyst quality indicators, including developmental stage, degree of blastocoel expansion, and ICM/TE morphology on birthweight and adverse neonatal outcomes. As such, it is expected that all measured neonatal outcomes were not statistically significant after stratifying by blastocyst quality and degree of expansion after adjusting for confounding factors in both fresh and frozen cycles.

Another explanation for the contradiction between studies is that the evaluation of blastocyst morphology is largely affected by subjective factors. A study has reported that the evaluation of blastocyst expansion by different embryologists is more consistent than the evaluation of TE/ICM morphology [28]. Interestingly, we found that the risk of LGA in the expanded, hatching, and hatched blastocyst group was significantly higher than that in the early and full blastocyst groups; however, significant differences were lost after adjusting for confounding factors. Fresh blastocyst transfer is mainly carried out on the day 5, and the majority of hatching and hatched blastocysts are cryopreserved on day 6. Therefore, the proportion of hatching and hatched blastocysts is extremely small in fresh cycles, which is one of the possible reasons for the lack of statistical difference in LGA. To further confirm the correlation between the degree of blastocoel expansion and LGA, the larger dataset from frozen-thawed cycles showed that birthweight (3336 g, 3389 g, 3399 g, and 3434 g) and LGA (18.7%, 21.0%, 22.3%, and 24.8%) increased gradually with advanced blastocyst expansion; however, there was no significant difference in birthweight (p=0.071), z score (p=0.056) and LGA (p=0.052) after adjusting confounding factors. As proportion of stage-6 blastocyst is also very small in frozen-thawed blastocyst transfer cohort (3.6%), a larger sample size may be required for stage-6 blastocyst cohort to detect significance for birthweight and LGA.

There are several advantages in this study. Firstly, we systematically analyzed the impact of blastocyst quality-related indicators, including ICM/TE morphology, degree of expansion, and developmental stage on birthweight and neonatal outcomes. Secondly, the newborn cohort resulting from the transfer of fresh and frozen-thawed blastocysts was included at the same time, which made the research results more generalizable. Thirdly, in the relevant studies so far, the sample size
was the largest and had strong statistical power. Finally, there was a high consistency in birthweight and neonatal outcomes between fresh and frozen-thawed blastocysts. The limitation of this study was its retrospective design, and sample size of stage 3 (n=150) and stage 6 blastocyst cohort (n=246) was relatively small.

**Conclusions**

No parameters of blastocyst quality were significantly correlated with birthweight and neonatal outcomes, including PTB, LBW, SGA, and LGA. Transfer of poor quality blastocysts is unlikely to have an adverse impact on the health of newborns. Transfer of stage-6 blastocyst can lead to extremely skewed sex ratio. However, the correlation between birthweight, LGA, and degree of blastocoel expansion needs to be further studied.

**Abbreviations**

assisted reproductive technology (ART)
inner cell mass (ICM)
trophoderm (TE)
preterm birth (PTB)
low birthweight (LBW)
large for gestational age (LGA)
small for gestational age (SGA)

**Declarations**

**Ethics approval and consent to participate**

This retrospective cohort study was approved by the Ethics Committee of Reproductive Medicine, Peking University Third Hospital (2021SZ-005). Informed written consent from patients was not required for this study.

**Consent for publication**

Not applicable

**Availability of data and materials**

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

**Competing interests**

The authors declare that they have no competing interests

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**Authors’ contributions**
J.Z.: data collection, statistical analysis, and manuscript preparation. Y.L, X.Z, S.L, X. Z, and J.L: data collection and analysis. P.L. and R.L.: study design

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