

# Does the sex ratio of singleton births after frozen single blastocyst transfer differ according to blastocyst morphology?

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## Research

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# Abstract

**Purpose:** To investigate the associations between morphological blastocyst parameters and the sex ratio (male:female) of singleton live births resulting from single-blastocyst frozen embryo transfer (FET) cycles.

**Methods:** This retrospective analysis included 1210 couples who underwent single-blastocyst FET of warmed day 5 or 6 blastocysts and achieved a singleton live birth between January 2015 and February 2019. The sex ratios in the different blastocyst groups were compared using the chi-square test. The associations of morphological blastocyst parameters, including the blastocoel expansion, inner cell mass and trophoctoderm grades, with the sex ratios of live births were analyzed using multiple regression models.

**Results:** The included female patients had an average age and body mass index of  $30.5 \pm 4.5$  years and  $23.6 \pm 3.2$  kg/m<sup>2</sup>, respectively. Blastocyst transfers occurred on day 5 in 783 cases (64.7%) and day 6 in 427 cases (35.3%). Among the day 5 FET cycles, 55.4% of resulting infants were male and 44.6% were female, while among the day 6 cycles, 54.6% of infants were male and 45.4% were female ( $P = 0.074$ ). Blastocysts quality was assessed according to morphological parameters, which was divided into high-quality (AA,AB,BB,BC) and poor-quality (AC,BC). High-quality blastocyst transfer was associated with a significantly higher sex ratio (60%, 383/638) relative to poor-quality transfer (49.7%, 284/572) ( $P < 0.001$ ). The sex ratio of births resulting from blastocyst transfer differed significantly with respect to the trophoctoderm grade ( $P < 0.001$ ). After adjusting for confounders, a grade B trophoctoderm was significantly associated with a higher sex ratio among singleton live births (odds ratio: 0.591, 95% confidence interval: 0.463–0.756,  $P < 0.001$ ; reference: grade C trophoctoderm). In contrast, the characteristics of blastocoel expansion and the score of the inner cell mass were not significantly associated with the sex ratio among singleton live births.

**Conclusions:** The sex ratio among singleton live births is affected by the quality of blastocysts. A grade B trophoctoderm is associated with a higher probability that a single-blastocyst FET cycle will result in a male infant.

## Introduction

Ideally, human assisted reproductive technology (ART) aims to achieve a single live birth. Following improvements in blastocyst culture systems and freezing technology<sup>[1,2]</sup>, single blastocyst transfer (SBT) is now considered the most effective means of avoiding multiple pregnancies subsequent to ART<sup>[3–6]</sup>. Gradually, SBT has become the preferred transfer strategy throughout the world<sup>[7–10]</sup>. However, blastocyst transfer has some potential limitations, including adverse effects such as a male-biased imbalance in the sex ratio and an increased incidence of monozygotic twinning (MZT)<sup>[11–13]</sup>.

The sex ratio at birth is often calculated as the proportion of males among all live births or the number of male births per 100 female births<sup>[14]</sup>. Dean et al. reported a significantly higher sex ratio at birth among babies born after SBT (54.1%) than among those born after cleavage-stage transfer cycles (49.9%)<sup>[15]</sup>. Another study determined that a higher sex ratio was independent of the fertilization method<sup>[16]</sup>. However, the details of the relationship between the characteristics of blastocysts and the sex ratio at birth remain unclear.

Blastocyst grading prior to embryo transfer usually occurs on day 5 or day 6 of culture. A detailed blastocyst scoring system is required to identify the blastocysts with the highest implantation potential. Static morphology remains the most commonly used embryo selection method worldwide. This system is used to grade blastocysts according to three different variables: blastocoel expansion, inner cell mass and trophectoderm<sup>[17]</sup>. Several studies have attempted to identify the individual contributions of these parameters to the implantation potential or live birth rate. Particularly, the trophectoderm morphology may be predictive of pregnancy outcomes related to single blastocyst transfer<sup>[18]</sup>.

Ebner et al. reported that the embryo sex ratio was skewed significantly in relation to the morphology. Male blastocysts had a 2.53 higher odds of receiving a trophectoderm quality score of A relative to female blastocysts<sup>[19]</sup>. All previous related studies were based on morphological parameters and embryo sex. To our knowledge, no previous study has explored the relationship between morphological blastocyst parameters and the sex ratio of singleton live births after single-blastocyst frozen embryo transfer (FET) cycles. The objective of this study was to investigate this association and resolve this gap in the literature.

## **Materials And Methods**

### **Study subjects**

This was a retrospective cohort study of 1210 couples (cycles) who underwent single-blastocyst FET at the Third Affiliated Hospital of Zhengzhou University from January 2015 to February 2019. Only the first FET cycle of each patient was analyzed to avoid repeated measures bias. Patients who gave birth to a singleton infant were included. Patients who underwent preimplantation genetic testing, used donor oocytes and donor sperm or achieved MZT were excluded. The data collection protocol for this study was approved by our Institutional Review Board.

### **Ovarian stimulation protocol**

Each female patient underwent a conventional ovarian hyper-stimulation procedure involving a gonadotrophin-releasing hormone agonist or antagonist. The physician adjusted the starting dose according to the patient's age, body mass index (BMI) and ovarian reserve. Ovarian follicle development was monitored based on serum estradiol and transvaginal ultrasonographic measurements. Oocytes

were retrieved transvaginally 36–38 hours after human chorionic gonadotrophin (Serono, Aubonne, Switzerland) administration when at least 40% follicles had reached or exceeded an average diameter of 18 mm as determined by ultrasound. The follicles were aspirated using a single-lumen needle attached to a syringe under transvaginal ultrasound guidance. The oocytes were then inseminated via conventional in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI).

## Laboratory protocol

Blastocyst morphology was evaluated according to the classification devised by Gardner and Schoolcraft<sup>[20]</sup> on day 5 or day 6 after insemination. As a general rule, the inner cell mass quality should be at least B to optimize cryosurvival. High-quality blastocysts were defined as those with a blastocoel grade >B3, inner cell mass grade A/B and trophectoderm grade A/B(AA, AB,BA,BB) Poor-quality blastocysts were defined as those with a blastocoel grade >B3, inner cell mass grade A/B and trophectoderm grade C(AC,BC)<sup>[21]</sup>.

Before vitrification, fully expanded blastocysts were collapsed artificially using a laser<sup>[22, 23]</sup>. After collapsing the blastocoel, the shrunken blastocyst was then vitrified using a Cryotop device (Kitazato BioPharma Co. Shizuoka, Japan) The media employed were vitrification and warming kits. For vitrification, the blastocysts were first equilibrated in solution I [7.5% v/v ethylene glycol (EG) and 7.5% v/v dimethyl sulfoxide (DMSO)] at room temperature for 10 min and then placed into vitrification solution II (15% v/v EG, 15% v/v DMSO and 0.5 M sucrose) for 1 min. Subsequently, Blastocysts were individually loaded onto Cryotops in a volume of <0.1µl and quickly plunged into liquid nitrogen. Warming was performed by placing the Cryotop in the thawing solution (1.0 M sucrose) for 1 minute at 37°C. Then the blastocyst was moved to the dilution solution (0.5 M sucrose) for 3 minutes at room temperature, followed by two steps in washing solution at room temperature for 5 minutes each. and transferred into a 50-µL droplet of culture medium (G<sub>2</sub>; Vitrolife) under mineral oil. Blastocysts were transferred 1–2 hours post-warming.

## Embryo transfer and clinical outcomes

FET was performed after preparation via hormone replacement treatment (HRT) or during a natural cycle. Vaginal or oral progesterone (Crinone, Merck Serono, Switzerland) was provided for luteal support. On day 6 of progesterone administration, a single vitrified blastocyst was selected for transfer based on morphology grading. All transfer procedures were directed by ultrasound guidance as previously described<sup>[24]</sup>. Only single blastocysts were selected for transfer. No blastocyst transfer was performed if the endometrial thickness was <7 mm. A clinical pregnancy was confirmed by the ultrasonographic visualization of an intrauterine gestational sac with fetal heart activity at 4 weeks after blastocyst transfer. In this study, the outcomes was a singleton live birth after an analyzed cycle. The sex ratio at birth was calculated as the proportion of males among all live births.

# Statistical analysis

All data analyses were performed using the SPSS 25.0 statistical software package. Continuous data are presented as mean and standard deviation. Differences between two categorical variables were analyzed using the chi-square test depending on the data distribution. A multivariate logistic regression analysis with adjustment for major covariates (age, BMI, duration of infertility, basal follicle stimulating hormone, endometrial thickness at transfer, type of insemination, type of infertility and day of embryo development at transfer) was used to assess whether the sex ratio of singleton live births was affected by various morphology parameters used for grading. The data are reported as adjusted odds ratios (OR) and 95% confidence intervals (95% CI). A  $P$  value  $<0.05$  was set as the threshold of statistical significance.

## Results

### Demographics and basic characteristics

A total of 1210 couples had singleton live births after their first FET cycles between 2015 and 2018. An analysis of the collected demographic and clinical data revealed that insemination was achieved via IVF in 855 cycles (70.7%) and via ICSI in 355 cycles (39.3%). Blastocyst transfer occurred on day 5 frozen in 783 cycles (64.7%) and on day 6 frozen in 427 cycles (35.3%). The analyzed cycles yielded 667 male infants and 543 female infants. The sex ratio after FET was 55.1% (667/1210). The baseline characteristics of the included treatment cycles are described in Table 1.

We further analyzed the differences in the sex ratios of live-born infants between day 5 and day 6 blastocyst FET cycles. Among the day 5 FET cycles, 55.4% (434/783) infants were male and 44.6% (349/783) were female, while among the day 6 FET cycles, 54.6% (233/427) of the resulting infants were male and 45.4% (194/427) were female. This difference was not statistically significant ( $\chi^2 = 0.083$ ,  $P = 0.074$ ; Figure 1).

As shown in Table 2, We evaluated the difference in trend between parameters with respect to embryo selection, including blastocyst quality (high vs.poor), high-quality (AA, AB, BA, BB), and poor-quality (AC or BC). high-quality blastocyst transfer (60%, 383/638) was associated with a significantly higher sex ratio relative to poor-quality transfer (49.7, 284/572) ( $\chi^2 = 13.139$ ,  $P < 0.001$ ).

To further assess the effect of the trophoctoderm and inner cell mass grades on the sex ratio among singleton live births, we stratified the data according to trophoctoderm grades of A, B and C and inner cell mass grades of A and B. We then calculated the sex ratios of singleton live births for the six groups (AA, AB, AC, BA, BB, BC) (Figure 2). The highest sex ratio (62.8% male) was observed in the BB group, while the lowest sex ratio (40.7%) was observed in the AC group. We identified significant associations of the sex ratio among singleton births with the morphological characteristics of blastocysts. Specifically, blastocysts with a lower inner cell mass grade and trophoctoderm B were significantly more likely to be male ( $\chi^2 = 7.047$ ,  $P = 0.019$  and  $\chi^2 = 7.921$ ,  $P = 0.029$ , respectively).

Finally, after the adjustment of potential confounders, the results of a multivariate analysis revealed a significant association of a trophoctoderm grade of B with a higher sex ratio among live births (OR: 0.591, 95% CI: 0.463–0.756,  $P = 0.000$  relative to grade C). In contrast, the blastocoel expansion and inner cell mass scores were not significantly associated with the sex ratio among live births. All ORs were adjusted for male/female age, body mass index, duration of infertility, basal follicle stimulating hormone, endometrium thickness on the day of transfer, insemination type, infertility type and blastocyst FET day. (Table 3)

## Discussion

Our study results indicate that implantation with high-quality blastocysts, rather than poor-quality blastocysts, during single-blastocyst FET cycles is associated with an increased sex ratio among live births. We further determined that only the trophoctoderm grade was associated with a significantly higher sex ratio among singleton live births. Similar observations were not observed for the blastocoel expansion and inner cell mass grades. Therefore, our findings demonstrate that a trophoctoderm grade of B is associated with a higher probability that a male infant will be born from a single-blastocyst FET cycle.

Single blastocyst transfer was designed to avoid the complications associated with multiple pregnancies and is thus becoming the preferred type of ART. The use of extended blastocyst culture conditions may favor the selection of male blastocysts for transfer, as male embryos are thought to exhibit greater preimplantation developmental rates. In this study, the sex ratio among single-blastocyst FET cycles was 55.1%, which was consistent with the ratios reported from previous studies. For example, a large nationwide longitudinal birth cohort study of 103,099 pregnancies in Japan reported a skewed sex ratio in favor of males after blastocyst transfer relative to spontaneous conception (OR: 1.095; 95% CI: 1.001–1.198). However, the sex ratios in the non-ART treatment and cleavage groups were slightly lower than or equivalent to the ratio in the spontaneous conception group<sup>[13]</sup>. A meta-analysis of 13 studies also suggested that blastocyst transfer was associated with a male-biased sex ratio when compared with cleavage-stage embryo transfer (OR: 0.89, 95% CI: 0.86–0.93)<sup>[25]</sup>. This observation may be attributable to the more frequent selection of male embryos for transfer, as male embryos develop more rapidly in vitro and thus may appear more viable at the blastocyst stage<sup>[26, 27]</sup>. We were interested to determine the mechanism underlying the observed phenomenon of sex deviation in offspring after blastocyst transfer. Accordingly, we retrospectively analyzed the potential effects of morphological parameters on the sex ratio after single-blastocyst FET.

Our study is the first to correlate various blastocyst parameter grades with the sex ratio among singleton live births after single-blastocyst FET cycles. We observed a significant increasing trend in the sex ratio in cases involving high-quality versus poor-quality blastocyst transfer, suggesting that the transfer of more advanced blastocysts may increase the proportion of male infants. We note that male blastocysts may grow more rapidly and receive better morphological scores than female blastocysts. These discrepancies may at least partially account for the skewed sex ratio at birth. Mohamad et al. reported a greater

likelihood of euploidy among blastocysts with good morphology scores and among embryos that progressed more rapidly to the blastocyst stage<sup>[28]</sup>. Therefore, differences in the observed sex ratios of live births subsequent to single-blastocyst FET cycles may also be attributable to blastocyst selection and transfer strategies.

We did not find that delayed blastulation had a significant effect on the newborn sex ratio, as similar values were observed for the groups that received day 5 and day 6 embryo transfers. Our results are consistent with those of a study published by El-Toukhy et al., which reported similar live birth rates after the transfer of day 5 and day 6 vitrified embryos<sup>[29]</sup>. Desai N et al. used a morphokinetics approach combined with preimplantation genetic screening test modelsto determine that embryos exhibiting delayed blastulation were more likely to exhibit aneuploidy<sup>[30]</sup>. The blastocyst morphology, which is based on blastocoel expansion, the trophoctoderm and the inner cell mass, must also be considered when evaluating the outcomes of FET. Initially, we observed an association between the degree of blastocoel expansion and the sex ratio among singleton live births in a small sample. However, we did not observe any association between these variables. Samer, et al. reported that sex-related differences in development are highly significant, such that male embryos were 2.6 times more likely to produce a grade 5 or 6 blastocyst than female embryos.<sup>[31]</sup> Our study differed from that study because we focused on the sex ratio at birth, rather than in the embryos. The functions of blastocoel expansion involve hatching from the zona pellucida, adhesion and invasion of the endometrium and establishment of maternal communication<sup>[31, 32]</sup>. In a recent study, Jing Zhao et al. confirmed that the degree of blastocoel expansion was a better predictor than the trophoctoderm or inner cell mass grade in terms of the likelihood of live birth after both single-blastocyst fresh transfer and FET cycles<sup>[33]</sup>. The degree of blastocoel expansion may be an essential factor in a successful pregnancy and should be prioritized when selecting a frozen blastocyst for transfer<sup>[34]</sup>.

Some previous studies have emphasized the importance of the inner cell mass with respect to the establishment of a pregnancy<sup>[35,36]</sup>. Irani et al. applied the same embryo grading criteria used in analysis to 477 preimplantation genetic testing confirmed single-euploid blastocyst FET cycles. These authors determined that the overall blastocyst quality and inner cell mass grade were the most effective predictors of a successful pregnancy and suggested the use of both parameters to facilitate the selection of high-quality euploid blastocysts for IVF transfer<sup>[28]</sup>. In contrast, Ahlström et al. emphasized the higher predictive strength of the trophoctoderm grade relative to the inner cell mass grade when selecting the optimal blastocyst for both fresh and frozen thawed cycles<sup>[37]</sup>. In our study, however, we did not find that the inner cell mass quality had a significant effect on the newborn sex ratio after single-blastocyst FET cycles.

In our analysis, the trophoctoderm grade was identified as the most important factor affecting the newborn sex ratio in after a single-blastocyst FET cycle. A significant association was observed between grade B trophoctoderm and sex ratio was observed among infants conceived via the transfer of blastocysts. In other words, the effect of blastocyst morphology on the sex ratio is only obvious when

blastocysts with grade B trophoctoderm are selected. The importance of the trophoctoderm during the critical implantation phase might be ascribed to its role as a promoter of hatching and endometrial invasion<sup>[38, 39]</sup>. According to Thomas, et al. the observed correlations of trophoctoderm performance with infant sex and the rates of implantation, pregnancy and pregnancy loss indicate that this measure will eventually be prioritized over the inner cell mass score<sup>[19]</sup>. Interestingly, we also demonstrated that after controlling for the inner cell mass, the trophoctoderm grade B was positively correlated with the highest sex ratio among singleton births. Disparities in morphological scores between male and female embryos are unlikely to reflect any difference in competence. However, these variations clearly emphasize the tendency of male embryos to reach the final stages of blastocyst development more rapidly than female embryos<sup>[30]</sup>.

This study has several strengths. First, all cycles and cryopreservation procedures were completed at a single institution according to a strict cycle protocol. Our embryologists scored several standardized transfer parameters using a consistent system. We also included only single-blastocyst FET cycles that resulted in singleton live births to enable us to control the within-transfer characteristics that are known to affect outcomes. Our aim was to isolate morphological blastocyst parameters as the exposure while regulating several other potential confounders to the extent possible. However, our study also has some limitations. First, this was a retrospective study of a relatively small number of patients treated at a single center. In the future, a study with the large sample size is needed to validate. Second, further analyses of blastocyst grading according to morphological parameters should investigate dynamic parameters time-lapse microscopy which allows the assessment of embryo morphodynamic patterns throughout preimplantation development. Third, our strategy required an inner cell mass grade of B or higher for optimal cryosurvival. Therefore, we were only able to evaluate the outcomes associated with an inner cell mass grade of A or B. Fourth, blastocyst transfer is more advantageous than cleavage-stage embryo transfer in terms of successful implantation and pregnancy rates. We prefer high quality blastocyst transfer to get a better clinical pregnancy rate. Although we determined that the transfer of high-quality blastocysts may skew the sex ratio in favor of male embryos, it is difficult to change perceptions regarding popular blastocyst transfer strategies.

## Conclusion

In summary, we have demonstrated that the morphological criteria associated with higher-quality blastocysts result in a higher sex ratio after single-blastocyst FET. However, we found that only the trophoctoderm grade was significantly associated with the sex ratio among offspring. Our findings may elucidate the underlying determinants of the skewed sex ratio after blastocyst transfer. Clinicians should be aware of the effects of certain protocols on sex distribution, given recent trends towards the increased use of blastocyst transfer. In future, we aim to identify additional markers of embryonic implantation potential, with the aim of maintaining the [equilibrium](#) of the sex ratio among offspring without affecting the pregnancy rate.

## Abbreviations

ART:assisted reproductive technology; SBT: single blastocyst transfer; MZT: monozygotic twinning; FET: frozen embryo transfer; BMI:body mass index; IVF: in vitro fertilization; ICSI: intracytoplasmic sperm injection; HRT:hormone replacement treatment; OR:odds ratios; 95%CI:95% confidence intervals; ICM: inner cell mass; TE: trophoctoderm.

## Declarations

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## Authors' contributions

Designer of the study: HL, SHC,DYH. Data acquisition and analysis: NL, XKZ, LS. Draft of the manuscript and interpretation: HL, Revision of the manuscript: XLW, SHC. All authors approved the final manuscript.

## Availability of data and materials

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

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## 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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## Tables

<b>Table 1.</b> Characteristics of included patients with singleton live births.	
Characteristic	Description
Number of cycles	1210
Mean female age (years, $\pm$ SD)	30.5 $\pm$ 4.5
Mean male age (years, $\pm$ SD)	31.58 $\pm$ 5.35
Mean female BMI ( $\pm$ SD)	23.6 $\pm$ 3.2
Mean duration of infertility (years, $\pm$ SD)	3. $\pm$ 2.6
Mean thickness of <b>endometrium</b> on the day of transfer (mm, $\pm$ SD)	9.55 $\pm$ 2.62
Basal follicle stimulating hormone(IU/L)	6.67 $\pm$ 2.42
Type of infertility	
Primary	541
Secondary	669
Type of insemination	
IVF	855
ICSI	355
Day of frozen embryo	
D5	783
D6	427

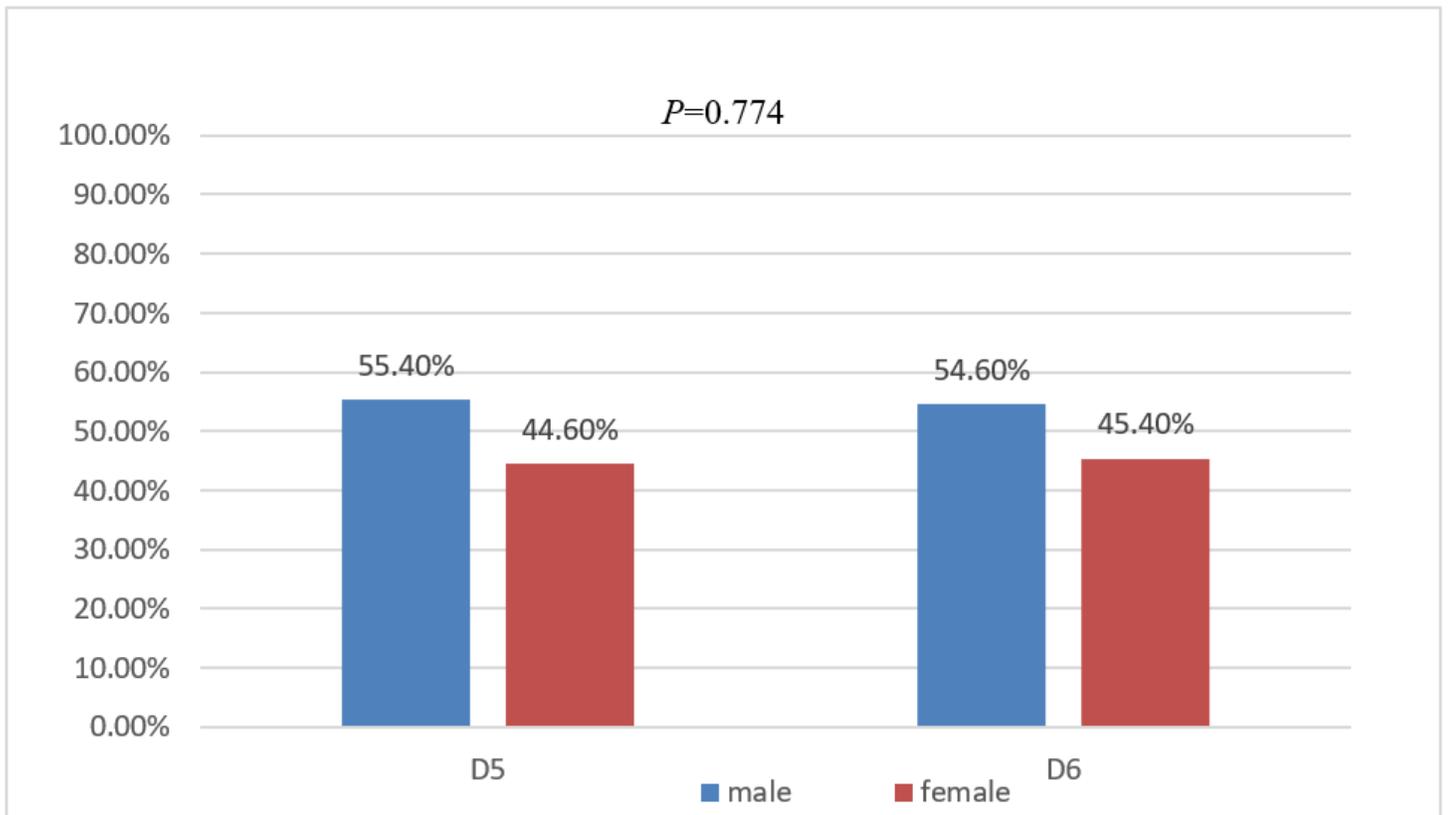
**Table 2.** Numbers of male born from cycles involving the transfer of blastocysts with different morphological parameters.

Blastocyst grading	Singletons (n)	Male (n)	Male percent (%)	<i>P</i> -value
High quality	638	383	60.0	0.000
Poor quality	572	284	49.7	
High quality				0.161
AA	52	26	50.0	0.343
AB	78	42	53.8	
BA	67	38	56.7	
BB	441	277	62.8	
Poor quality				
AC	27	11	40.7	0.343
BC	545	273	50.1	

**Table 3.** Associations of blastocyst morphological parameters with sex ratio.

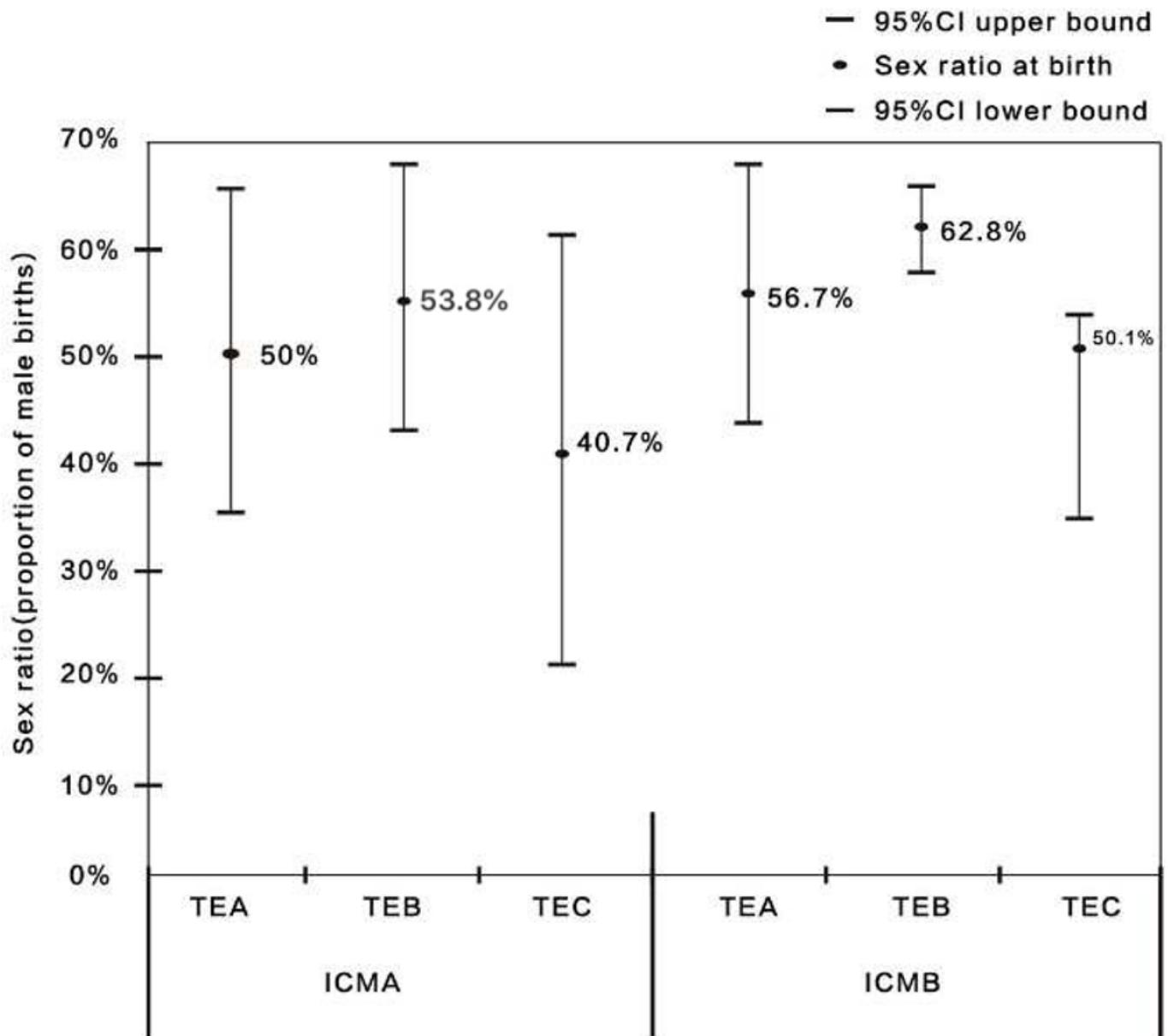
Parameter	Crude OR (95% CI)	<i>P</i>	Adjusted OR (95% CI)	<i>P</i>
Blastocyst expansion phase				
4	1.604 (0.889–2.893)	0.117	1.607 (0.878–2.942)	0.124
5	1.757 (0.866–3.566)	0.119	1.710 (0.838–3.489)	0.140
6	Reference		Reference	
Inner cell mass				
A	1.398 (0.975–2.006)	0.069	1.430 (0.993–2.058)	0.055
B	Reference		Reference	
Trophectoderm				
A	0.735 (0.482–1.122)	0.153	0.710 (0.460–1.096)	0.122
B	0.591 (0.463–0.756)*	<0.001	0.582 (0.451–0.753)*	<0.001
C	Reference		Reference	
Adjusted for male/female age, body mass index, duration of infertility, basal follicle stimulating hormone, endometrium thickness on the day of transfer, insemination type, infertility type and blastocyst FET day . OR, odds ratio; CI, confidence interval.				
* <i>P</i> < 0.05 was considered statistically significant; “B” vs. “C” trophectoderm				

# Figures



**Figure 1**

Sex of infants resulting from blastocyst frozen transfer on day 5 or day 6.



**Figure 2**

Sex ratio at singleton births following frozen SBT by different grade of trophoderm (TE) and inner cell mass (ICM). CI, confidence interval.