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## Original Article

## Initial serum HCG levels are higher in pregnant women with a male fetus after fresh or frozen single blastocyst transfer: A retrospective cohort study

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## ABSTRACT

**Objective:** Substantial previous studies have almost reached an agreement on the gender effect on maternal serum human chorionic gonadotropin (MsHCG) in and after the late first trimester of pregnancy. However, there is little knowledge of the sex-related difference in MsHCG level at the preliminary stage of pregnancy. The purpose of this study is to reveal this difference in women after fresh or frozen single blastocyst transfer (SBT).

**Materials and methods:** A total of 252 fresh SBT cycles and 1486 frozen-thawed SBT cycles collected between June 1, 2014 and May 30, 2017 were retrospectively analyzed in our center. Patients with MsHCG level  $\geq 5$  IU/L on day 11 after transfer, achieving a singleton intrauterine pregnancy and subsequent live birth were included. We compared MsHCG levels between women gave birth to a male neonate and those gave birth to a female one in fresh or frozen SBT cycles, respectively.

**Results:** A total of 136 neonates including 57 females and 79 males were born following fresh SBT. The male-female ratio was 1.39:1. The average MsHCG level of male fetuses was higher than that of female fetuses on day 11 after transfer ( $549.82 \pm 253.24$  IU/L versus  $439.03 \pm 198.41$  IU/L,  $P < 0.05$ ). Correspondingly, a total of 431 infants was born after frozen SBT, containing 188 females and 243 males. The male-female ratio was 1.29:1. Initial MsHCG level remained higher in women with a male neonate than the counterparts with a female neonate ( $894.43 \pm 622.17$  IU/L versus  $758.05 \pm 624.33$  IU/L,  $P < 0.05$ ). It was also found the pregnant women following frozen-thawed SBT exhibited higher initial MsHCG level than those following fresh SBT in whether male-bearing or female-bearing gestations.

**Conclusions:** MsHCG levels are higher in pregnant women with a male fetus than those with a female one on day 11 after fresh or frozen SBT. A sex-specific response to the stress in the process of *in vitro* embryo culture was suggested.

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## Introduction

Blastocyst stage embryo transfer (BT) is becoming more and more common in both *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) cycles [1]. The advantages of BT include the better endometrium-embryo synchronization and a further selection of embryos with higher implantation potential [2]. Moreover, single blastocyst transfer (SBT) has become an effective

strategy to decrease the number of embryos transferred and sequentially minimize the risk of multiple gestations [3]. Nevertheless, the disadvantages of BT also attract full attention. Besides the high occurrence of embryo transfer cancellation, monozygotic twinning, and epigenetic mutations in the fetus, a biased sex ratio in favor of male offspring is one broadly concerned side-effect, especially in the perspective of demographers. Although several articles challenged this finding [4], most studies consistently reported a higher occurrence of male infants after BT [5–8]. The sex ratio skew was thought to result from more advanced growth rates of male embryos compared to females at different stages of gestations [9], which was same as the reports in animals [10,11].

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Maternal serum human chorionic gonadotropin (MshCG) was also reported to be significantly related to the fetal sex ratio [12], which is secreted by the human pre-implantation embryos and can be detected in maternal blood approximately 6–8 days after fertilization [13,14]. And post-implantation HCG is primarily secreted by the syncytiotrophoblast cells, with levels increasing throughout the first trimester of pregnancy to peak around 10 weeks [15,16]. HCG provides the best sensitivity and specificity for the earliest detection of normal or aberrant pregnancies [17], which is widely used in current clinic [18]. And HCG secretion activity might be indicative of embryo developmental potential and function [19]. Moreover, MshCG level was firstly demonstrated higher in women bearing a female fetus than those with a male in the third trimester of gestation [12]. Subsequently, series studies confirmed the gender effect on MshCG levels in the second trimester [20] and even in the late first trimester of pregnancy [21], all of them consistently suggested an increased MshCG level in the presence of a female fetus, which was unexpectedly opposite to the scenario that male embryos develop faster than female embryos. This phenomenon presented was explicated by the differential activity of the fetal hypothalamic-hypophyseal-gonadal axis (HHGA) [22]. However, the gender effect on MshCG production was obscure in the preliminary stage of pregnancy when MshCG is drawn for the biochemical pregnancy evaluation. As we know, gonads are not generated at that time [23], whether initial serum HCG levels in women carrying a female baby are still higher than those carrying a male one was controversial [24,25].

Our previous analysis of obstetric and perinatal outcomes in women with different initial MshCG levels after SBT indicated a female bias in the low MshCG group [26]. To further clarify the sex-related difference in the preliminary stage of pregnancy, initial MshCG levels on day 11 after fresh SBT or frozen SBT resulting in a male or a female neonate were investigated.

## Materials and methods

### Patients

A total of 252 fresh SBT cycles and 1486 frozen-thawed SBT cycles carried out between June 1, 2014 and May 31, 2017 at our fertility center were retrospectively analyzed. Cycles with positive serum HCG ( $\geq 5$  IU/L) detected on day 11 after SBT in our institute and harvesting singleton live birth subsequently were included. Any cycles with an ectopic pregnancy or a monozygotic twin pregnancy were excluded. A flowchart of patients recruiting are showed in Fig. 1.

### Stimulations in fresh cycles

Common stimulation protocols were implemented by clinicians in our center based on the individual ovarian reserve and response, including long down-regulation protocols, antagonist protocols, and clomiphene-based mild stimulation protocols. HCG or gonadotropin-releasing hormone agonist was used as a trigger

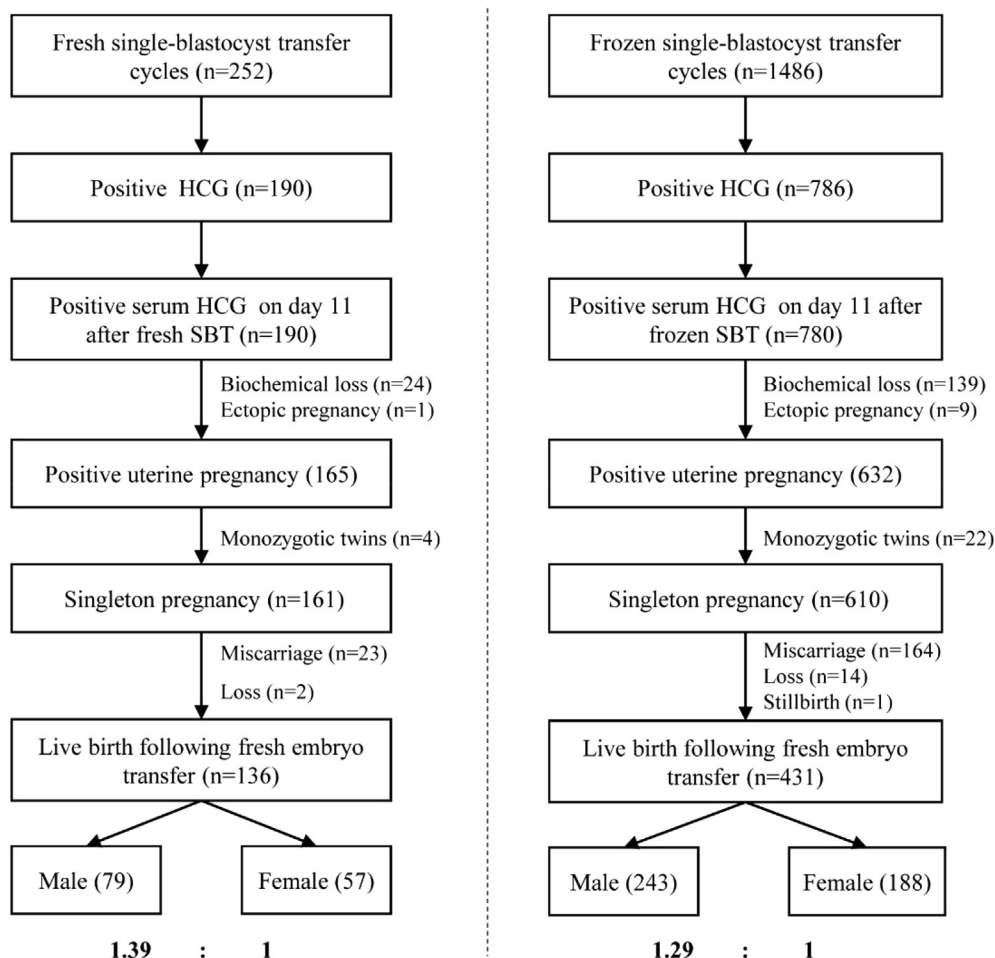


Fig. 1. Flowchart of cycles included in the study.

when there were two leading follicles  $\geq 18$  mm in diameter. The dosage of drugs might be slightly adjusted based on clinical considerations such as the growth of follicles, variation of hormone levels, and risk of ovarian hyper-stimulation syndromes (OHSS).

#### *Embryo culture and fresh blastocyst transfer*

Ovum pick-up (OPU) was conducted 36 h post HCG injection guided by the vaginal ultrasound. Insemination or ICSI was performed 3–4 h after OPU. Generally, ICSI was taken slightly later than insemination in order to observe fertilization simultaneously. In the first three days, the embryos were individually cultured in Quinn's Advantage Cleavage Medium (SAGE, US) drops with 10% (v/v) serum protein substitute (SPS) (SAGE, US), and on day 3 they were change to Quinn's Advantage Blastocyst Medium (SAGE, US). The blastocysts were graded based on the Gardner system [27]. The embryos with inner cell mass (ICM) and trophoctoderm (TE) was graded as CC would not be transferred or frozen. Available blastocysts on day 5 were transferred under the ultrasound guidance. Surplus embryos not selected for transfer or formation on day 6 were cryopreserved.

#### *Embryo cryopreservation*

All blastocysts were induced to collapse blastocoel with laser before cryopreservation. Vittrification was carried out with standard two steps. Firstly, immersed shrinkage blastocyst in the equilibration medium containing 7.5% (v/v) ethylene glycol (EG), 7.5% (v/v) dimethyl sulfoxide (DMSO), and 5% (m/v) human serum albumin (HSA) for 5 min at room temperature. And then transferred it to the vittrification medium containing 15% (v/v) EG, 15% (v/v) DMSO, 5% (m/v) HSA, and 0.5 M sucrose for 45–60 s. An opened cryotop carrier device (Kitazato, Japan) was used for embryo cryopreservation.

Embryo thawing was performed sequentially in solutions containing 1 M, 0.5 M, and 0 M sucrose with 5% (m/v) HSA. All the warming steps were carried out at room temperature except for the first one, which was conducted at 37 °C. Each blastocyst underwent laser-assisted hatching [28] immediately after thawing (ZILOS-tk laser, Hamilton Thorne, US) and transferred under the ultrasound guidance 2–3 h later.

#### *Endometrial preparation in frozen cycles*

Endometrium was prepared by either the natural protocol for the patients with regular ovulation or the artificial protocol for patients with ovulation disorder. In the natural protocol, follicle monitoring was implemented, and embryo transfer was carried out on day 5 after ovulation if the endometrial thickness exceeded 7 mm. In the artificial protocol, the patients received 4, 6 and 8 mg oral oestradiol per day successively. Progesterone was started when the endometrial thickness reached to 7–8 mm, and embryo transfer was done on day 6 after progesterone injection.

#### *Luteal phase support*

Luteal phase support (LPS) after fresh SBT or frozen SBT was achieved with oral dydrogesterone tablets (20 mg, bid) and intra-vaginal progesterone (90 mg Crinone gel, qd; or 300 mg Utrogestan, tid) for two weeks until positive MsHCG test and continued until three months of gestation. No HCG products were used for LPS in fresh SBT or frozen SBT cycles.

#### *HCG detection*

MsHCG measurement was performed on 11 days after fresh SBT or frozen SBT using chemiluminescent microparticle immunoassay (CMIA) technology with Architect-Total HCG analytical system (Abbott, US). The same standard assay was used for all included cycles, and quality control of the accuracy was carried out every day.

#### *Statistical analysis*

Statistical analyses including multivariable logistic regression analysis were performed using SPSS version 24 (IBM company, USA). Quantitative data were presented as the mean and standard deviation (mean  $\pm$  SD), and the unpaired two-tailed Student's *t*-test was performed to compare the difference between two gender groups. Categorical variables were displayed as frequencies with percentages in the rounded brackets, and the Chi-squared test and Fisher's exact test were used as appropriate to evaluate the significance. A *P* value  $< 0.05$  was considered to be statistically significant. Patient characteristics were tabulated by the gender of live birth, and MsHCG levels were comparatively exhibited with histograms.

#### **Results**

In a total of 252 fresh SBT cycles, 190 resulted in a positive HCG value on day 11 after transfer. Excluding cycles with multiple gestations, ectopic pregnancies and miscarriages, 136 live birth cycles were available for final analysis, in which containing 79 male neonates and 57 female neonates. The sex ratio of male to female was 1.39:1. Comparatively, a total of 431 live birth cycles with a singleton harvested from 1486 frozen SBT cycles. Of them, 188 were males and 243 were females. The sex ratio of male to female was 1.29:1 (Fig. 1). Patients demographics and embryo parameters in fresh cycles and frozen cycles are individually listed in Tables 1 and 2. There was no significant difference in baseline characters like maternal age, paternal age, BMI, embryo quality, and the prevalence of first embryo transfer between two gender groups, whether in fresh or frozen SBT cycles. Moreover, the morphological scores on the inner cell mass (ICM), trophoctoderm (TE), and the expanding status of the blastocysts transferred were also comparable. However, in frozen SBT cycles, more male-bearing women had a history of conceptions (previous gravidity  $\geq 1$ ) and more female-bearing women accepted ICSI-derived embryos transfer ( $P < 0.05$ ) (Table 2). The gravidity and ICSI proportion between the female live birth group and the male live birth group were similar in fresh SBT cycles.

In fresh SBT cycles, the distribution of MsHCG levels on day 11 after transfer is presented in Fig. 2a. The mean MsHCG level was  $549.82 \pm 253.24$  IU/L in the male-bearing group, which was significantly higher than  $439.03 \pm 198.41$  IU/L in the female-bearing group ( $P < 0.05$ ) (Fig. 2b). Next, initial MsHCG levels were identically analyzed in live birth cycles after frozen SBT, the distribution of MsHCG concentrations on day 11 is displayed in Fig. 3a. The mean value showed the same sex-related difference, which was  $894.43 \pm 622.17$  IU/L in the male-bearing group, higher than  $758.05 \pm 624.33$  IU/L in the female-bearing group ( $P < 0.05$ ) (Fig. 3b). Furthermore, this trend remained significant ( $P < 0.05$ ) after adjusting the gravidity, fertilization method, maternal age, BMI, embryo transfer cycle, and embryo quality. Moreover, the average MsHCG levels between fresh SBT cycles and frozen ones were also compared in the perspective of gender. The results showed that frozen SBT induced higher initial MsHCG than fresh SBT in whether male-bearing or female-bearing pregnancy (Fig. 4).

**Table 1**

Demographic characteristics and embryo parameters of women achieving a male neonate or a female one after fresh single blastocyst transfer (n = 136).

Characteristics	Female live births (N = 57)	Male live births (N = 79)	P value <sup>b</sup>
Maternal age (years)	30.88 ± 3.87	30.71 ± 3.81	NS
Paternal age (years)	34.56 ± 5.90	33.72 ± 5.45	NS
BMI (kg/m <sup>2</sup> )	21.29 ± 2.40	20.89 ± 2.55	NS
Endometrial thickness (mm) <sup>a</sup>	11.91 ± 2.34	11.77 ± 2.41	NS
Infertility duration (years)	3.32 ± 2.51	3.16 ± 2.08	NS
First ET cycle, n (%)	53 (92.98)	74 (93.67)	NS
First gravidity, n (%)	24 (42.10)	32 (40.51)	NS
Oocytes number	13.67 ± 3.36	13.06 ± 3.67	NS
ICSI, n (%)	14 (24.56)	15 (18.99)	NS
High quality embryos transferred, n (%)	53 (92.98)	75 (94.94)	NS
Day 5 blastocysts transferred, n (%)	57 (100)	79 (100)	NS
Blastocyst morphological scores			
Expanding status, n (%)			NS
3	4 (7.02)	9 (11.39)	
4	53 (92.98)	69 (87.34)	
5	0 (0.00)	1 (1.27)	
ICM grade, n (%)			NS
A	31 (54.39)	46 (58.23)	
B	26 (45.61)	33 (41.77)	
C	0 (0.00)	0 (0.00)	
TE grade, n (%)			NS
A	20 (35.09)	36 (45.57)	
B	33 (57.89)	39 (49.37)	
C	4 (7.02)	4 (5.06)	

BMI, body mass index; OPU, ovum pick-up; ET, embryo transfer; ICSI, intracytoplasmic sperm injection; ICM, inner cell mass; TE, trophectoderm. NS, not significant.

<sup>a</sup> Endometrial thickness on the day of HCG injection.<sup>b</sup> P < 0.05 indicated a significant difference.**Table 2**

Demographic characteristics and embryo parameters of women achieving a male neonate or a female one after frozen single blastocyst transfer (n = 431).

Characteristic	Female live births (N = 188)	Male live births (N = 243)	P value <sup>b</sup>
Maternal age at transfer (years)	33.02 ± 4.13	33.29 ± 4.10	NS
Paternal age at transfer (years)	35.59 ± 5.46	36.39 ± 5.69	NS
BMI (kg/m <sup>2</sup> )	20.86 ± 2.57	21.32 ± 2.85	NS
Endometrial thickness (mm) <sup>a</sup>	9.68 ± 1.54	9.68 ± 1.59	NS
First ET cycle, n (%)	58 (30.85)	70 (28.81)	NS
First gravidity, n (%)	58 (30.85)	50 (20.58)	<0.05
Oocytes number	15.16 ± 7.58	14.28 ± 7.26	NS
ICSI, n (%)	76 (40.43)	73 (30.04)	<0.05
High quality embryos transferred, n (%)	119 (63.30)	171 (70.37)	NS
Day 5 blastocysts transferred, n (%)	98 (52.13)	127 (52.26)	NS
Blastocyst morphological scores			
Expanding status, n (%)			NS
3	7 (3.72)	7 (2.88)	
4	165 (87.77)	213 (87.65)	
5	15 (7.98)	17 (7.00)	
6	1 (0.53)	6 (2.47)	
ICM grade, n (%)			NS
A	68 (36.17)	91 (37.45)	
B	117 (62.23)	150 (61.73)	
C	3 (1.60)	2 (0.82)	
TE grade, n (%)			NS
A	26 (13.83)	51 (20.99)	
B	96 (51.06)	122 (50.21)	
C	66 (35.11)	70 (28.81)	

BMI, body mass index; OPU, ovum pick-up; ET, embryo transfer; ICSI, intracytoplasmic sperm injection; ICM, inner cell mass; TE, trophectoderm. NS, not significant.

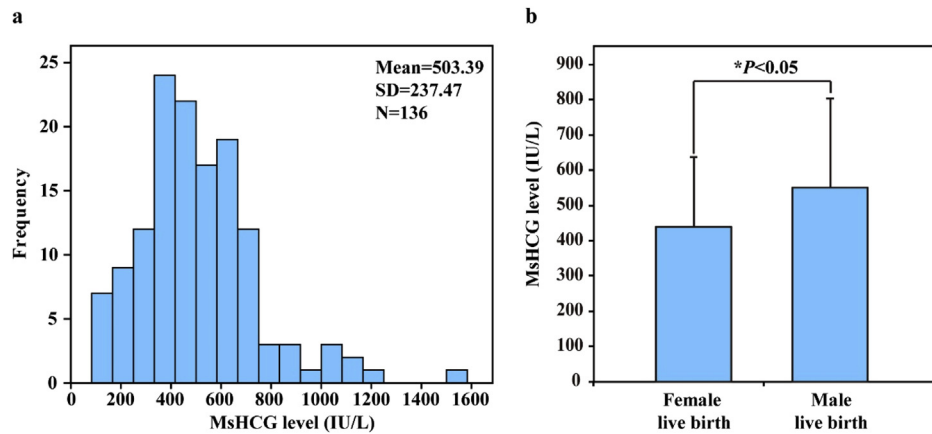
<sup>a</sup> Endometrial thickness on the day of progesterone initiation.<sup>b</sup> P < 0.05 indicated a significant difference.

## Discussion

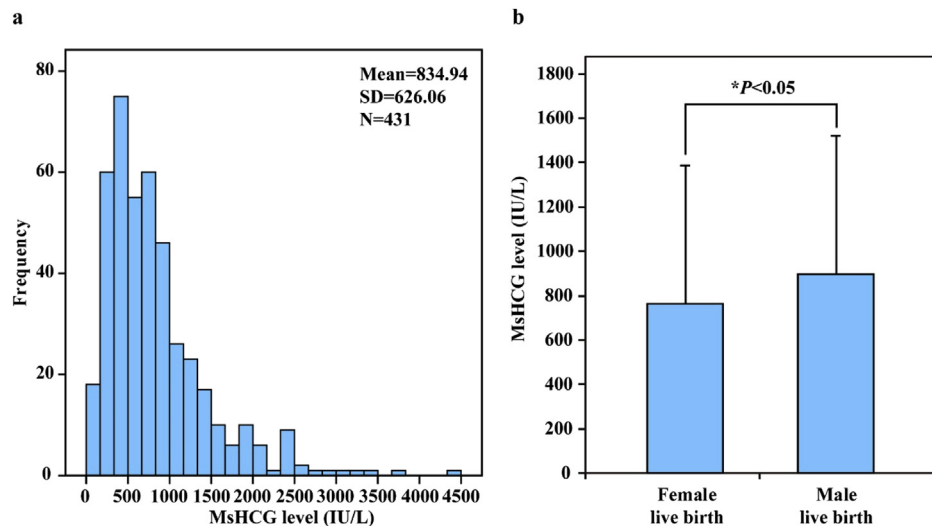
In this study, the association of fetal gender with MshCG levels on day 11 after fresh or frozen SBT was evaluated. It was found that MshCG level was affected by fetal gender in the very early stage of pregnancy, whether in fresh or frozen SBT cycles. The serum HCG concentration was significantly higher in women carrying a male fetus compared with those carrying a female one. This study supplementally revealed a specific sex-related MshCG difference in the

preliminary stage of pregnancy, which is opposite to the trend in and after the later first trimester of gestation. Our findings also confirmed previous reports that frozen SBT led to higher initial MshCG levels than fresh SBT [29], but this study further revealed this trend was not sex-related.

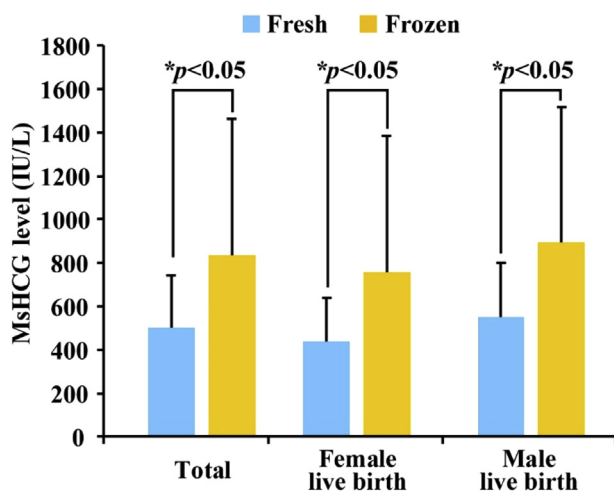
Series previous articles demonstrated the gender effect on MshCG level in the third trimester [12], second trimester [20] and even late first trimester of pregnancy [21], all of them suggested an increased MshCG level in the presence of a female fetus. Yaron et al.



**Fig. 2.** Distribution of maternal serum human chorionic gonadotropin (MhCG) levels on day 11 following fresh single blastocyst transfer (a) and initial MhCG comparison between two genders of live birth resulted from fresh single blastocyst transfer (b).  $P < 0.05$  indicated a significant difference.



**Fig. 3.** Distribution of maternal serum human chorionic gonadotropin (MhCG) levels on day 11 following frozen single blastocyst transfer (a) and initial MhCG comparison between two genders of live birth resulted from frozen single blastocyst transfer (b).  $P < 0.05$  indicated a significant difference.



**Fig. 4.** Initial maternal serum human chorionic gonadotropin (MhCG) levels are higher in frozen single blastocyst transfer cycles without fetal sex difference.  $P < 0.05$  indicated a significant difference.

investigated the MhCG level three weeks after fertilization in both single-embryo and multiple-embryos transfer cycles and found it remained higher in female fetus pregnancy [24]. These studies suggested that fetal gender had a significant effect on MhCG level, particularly in the late stage of gestation. The reason for this phenomenon is still obscure, and no satisfactory explanation has been provided. Obiekwe et al. put forward a hypothesis that the sex-related difference in MhCG level was the result of the differential activity of the fetal HHGA, which influenced the production and consumption of HCG through controlling the secretion of fetal sex hormone [22,24]. Although Gol et al. challenged the central role of HHGA in this scenario [30], some evidence of sex-induced difference on sex hormones were found. For example, Higher estradiol level was reported among women with a female fetus compared to those with a male fetus, lower progesterone and testosterone concentrations were revealed in mothers of girls than those of boys [31–34]. Whether the gender of fetus significantly influences the level of sex hormones to control HCG secretion or not still need further study. However, even if the assumption based on fetal hormones adjustment is evidenced, it seems too early to account for the MhCG difference just on day 11 after SBT, when the essential organs for hormones production have not yet developed.



One possible reason for the sex-related MSHCG differences in the preliminary stage of pregnancy was resulting from the faster developmental rate of the male embryo *in vitro* culture, which was reported by numerous studies [35,9]. And it is also the potential reason causing a male-biased sex ratio after fresh or frozen SBT in this study (Fig. 1). The sex ratio skew to male probably occurred simultaneously with the phenomenon that MSHCG levels were higher in the male bearers. Nevertheless, a retrospective analysis of the blastocyst morphological scores in both groups showed no significant difference in expanding status, grade of ICM and TE on the day of transfer (Tables 1 and 2), which was beyond our expectation. Therefore, the hypothesis of different developmental rate leading to significant initial MSHCG difference was challenged. It would happen only if male preimplantation embryos began to develop faster than female embryos after the day of transfer, as implied in one previous study [6]. This difference in growth rate may result from gender effect on embryo-maternal cross-talk, which is essential for embryonic development and implantation [36,37]. Faster growth of male embryos induces more trophoblast cells than female embryos and leads to advanced implantation in pregnancy, which will cause premature HCG secretion and the accumulation of MSHCG on the detection day, as MSHCG are dynamically increased with the progression of pregnancy [16].

Another more possible explanation for the MSHCG difference was originating from the distinction in the chromosomal constitution of the male embryo and the female one. As we know, Y chromosome exists only in the male embryo, and the female embryo possesses two active X chromosomes throughout preimplantation development [38]. It was supposed that there was a specific dosage compensation mechanism during embryo preimplantation stage before X-chromosome inactivation (XCI) established. During this process, X-linked genes were downregulated progressively [39]. These complex biological events modulate the specificity of the female embryo on the metabolism, gene transcription and protein translation [40], which may cause physiological variations such as an increased glucose utilization rate and a higher vulnerability to stress in the female embryos compared with the male ones cultured *in vitro* [41]. This scenario was evidenced by that glucose-6-phosphate dehydrogenase (G6PD) and phosphoglycerate kinase (PGK), two critical glycolysis-related enzymes, exhibited higher expression and activity in female embryos [42,43]. Excess glucose exposure shifts the glucose metabolism of the female conceptus to the pentose phosphate pathway, whose secondary metabolites are probably toxic to the growth and even survival of fetuses [44]. Most commercial blastocyst and cleavage embryo culture mediums contain a high level of glucose as the primary energy source [45,46], which is possibly detrimental to the viability of female embryos and results in their vulnerability to the environmental stress [47]. In consideration of HCG secreted by the human preimplantation embryo serves as a critical marker for embryo viability [48], low HCG level in the female fetus is probably one physiological feedback to the adverse condition in the process of *in vitro* culture.

In conclusion, our study revealed that MSHCG levels are higher in pregnant women with a male fetus than those with a female fetus at the preliminary stage of gestation after whether fresh or frozen SBT, which is opposite to the findings reported in and after the later first trimester of pregnancy. To our knowledge, this was the first study revealing a specific mode of gender effect on the initial MSHCG levels in both fresh and frozen SBT cycles. Unfortunately, MSHCG at that time is difficult to be applied individually as a sensitive prospective gender selection criterion, and it also cannot be intervened even if the sex ratio skews indicated by MSHCG levels. However, the finding of MSHCG difference in both gender groups suggested a sex-specific response of human embryos to the

stress in the process of assisted reproductive technology (ART), which arouses cautions on the health of neonates from ART in the perspective of gender. Moreover, it also reminds the embryologists of optimizing the culture conditions and embryo manipulation *in vitro* to maintain the embryos a natural-mimic developmental environment and to reduce the possible sex-related difference.

## Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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