Frozen blastocysts: Assessing the importance of day 5/day 6 blastocysts or blastocyst quality

YAN JIANG, GE SONG, XU-HUI ZHANG, SUI-BING MIAO and XIAO-HUA WU

The Center for Reproductive Medicine and Infertility, The Fourth Hospital of Shijiazhuang, Shijiazhuang Obstetrics and Gynecology Hospital Affiliated to Hebei Medical University, Shijiazhuang, Hebei 050011, P.R. China

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Abstract. The aim of the present study was to analyze the high-quality blastocyst (HB) rate in all embryo frozen cycles and investigate the pregnancy outcomes for day 5/day 6 (D5/D6) blastocysts with respect to the blastocyst quality in programmed single vitrified-warmed blastocyst transfer (SVBT). We performed a retrospective study comparing D5/D6 HBs in *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) for all blastocyst frozen cycles. Patients were <35 years at the oocyte collection in their first fresh cycle without fresh transfer. A total of 1,560 IVF/ICSI cycles and 5,328 blastocysts were analyzed. The IVF HB rate was higher than that of ICSI (52.7% vs. 42.6%; P<0.05). The D5 HB rate was much higher than the D6 HB rate (61.6% vs. 29.4%; P<0.05). There were 22.4% (349/1,560) cycles that only had D6 blastocysts, of which IVF cycles were lower than ICSI (19.8% vs. 28.5%; P<0.05). The clinical pregnancy rate and implantation rate in the D5 group were significantly higher than these rates in the D6 group (57.4% vs. 46.2%, 58.9% vs. 47.3%; P<0.05). However, the clinical pregnancy rate and implantation rate of the D5 HBs were not significantly different from those of the D6 HBs (60% vs. 54.5%, 62% vs. 56.3%; P>0.05). In conclusion, the fertilization method (IVF/ICSI) directly influences the HB rate and blastocyst development rates. When we controlled for patient age, transfer frequency, and endometrium on day 5, it was not the development stage (D5/D6), rather the transfer blastocyst quality that played an important role in pregnancy outcomes.

Correspondence to: Dr Yan Jiang, The Center for Reproductive Medicine and Infertility, The Fourth Hospital of Shijiazhuang, Shijiazhuang Obstetrics and Gynecology Hospital Affiliated to Hebei Medical University, 206 Zhong-Shan-Dong, Shijiazhuang, Hebei 050011, P.R. China
E-mail: 13932117427@163.com

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Introduction

Extended culture leading to embryo transfer at the blastocyst stage is considered a major advance in *in vitro* fertilization (IVF) as it has been shown to result in higher live birth rates in comparison to cleavage-stage embryo transfer (1,2). Transferring blastocysts is therefore perceived as the best option for elective single-embryo transfer (eSET), by reducing the risk of multiple pregnancies (3,4) without compromising live birth rates. With advances in cryopreservation techniques, improved implantation rates have been achieved, and pregnancy rates after frozen embryo transfer (FET) are at least comparable with those after the transfer of fresh IVF embryos (5). How to select single-blastocyst transfer for FET to reduce the time to successful pregnancy is very important. Thus, it is crucial to determine whether blastocyst developmental stage [day 5 (D5)/day 6 (D6)] or blastocyst quality is more important for a successful pregnancy.

The comparison of pregnancy outcomes between D5 and D6 blastocysts remains controversial. Some reports have concluded that the blastocyst development time is crucial and suggest that D5 is more superior than D6 in terms of implantation rate and live birth rate (6,7). However, other reports have shown that the clinical outcomes between D5 and D6 cryopreserved blastocyst transfers did not significantly differ (8,9), while some studies recommend that blastocyst quality is an important factor that affects the pregnancy outcomes of the vitrified-warmed cycles (10,11).

Previous relevant studies only performed simple statistical analyses of the clinical outcomes of vitrified-warmed cycles (6-11). However, all frozen D5/D6 blastocysts came from IVF and intracytoplasmic sperm injection (ICSI) fresh cycles. The present study was the first to consider the D5/D6 high-quality blastocyst rate of fresh cycle without embryo transfer and the ratio if only D6 have blastocysts. The present study also aimed to analyze the pregnancy outcomes of D5/D6 blastocysts with respect to the blastocyst quality in programmed single vitrified-warmed blastocyst transfer (SVBT).

Patients and methods

Patients. This was a retrospective study carried out at The Center for Reproductive Medicine and Infertility, The Fourth Hospital of Shijiazhuang, China from March 2017

to May 2020. In total, 1,560 (IVF, 1,100 and ICSI 460) all blastocyst frozen cycles were analyzed and 1,161 SVBT cycles (D5 975 and D6 186) were included in the study. The criteria for allocating patients to IVF and ICSI were male semen factors. The women were given IVF protocol in the first assisted reproductive technology (ART) treatment cycle unless accompanied by severe male-factor infertility. Otherwise ICSI treatment was performed. Patients included in the analysis were <35 years at the oocyte collection in their first fresh cycle without fresh embryo transfer, and were undergoing their first autologous FET cycle. The Fourth Hospital of Shijiazhuang Ethics Committee approved (approval no. 20170063; approval date, January 5, 2017) this study.

Blastocyst culture and scoring. Cumulus-oocyte-complexes (COC) were isolated from follicular fluid, rinsed in G-IVF™ medium (VitroLife, Sweden) transferred to 0.5 ml G-IVF™ medium and cultured in an incubator under 5% O_2, 6% CO_2, and 89% N_2. Sperm was used for either routine IVF insemination or ICSI procedure using a standard method as described by Jiang *et al* (12). Insemination were performed 38-40 h after trigger. Fertilization was identified by the presence of two pronuclei approximately 16-19 h after insemination or microinjection. On day 3, the embryos were transferred into G-2 culture medium in group culture (Vitrolife). In the morning of D5 or D6, blastocysts were scored by two experienced embryologist using the system of Gardner and Schoolcraft (13). On day 5, embryos at the morula or early blastocyst stage were left in culture for 1 day more. For blastocysts graded as 3-6 (i.e., full blastocysts onward), the development of the inner cell mass (ICM) was assessed as follows: A, tightly packed, many cells; B, loosely grouped, several cells; or C, very few cells. The trophectoderm (TE) was assessed as follows: A, many cells forming a cohesive epithelium; B, few cells forming a loose epithelium; or C, very few large cells. Blastocysts with a score ≥3, including those with grades BC, CB were selected on day 5 and day 6 for vitrification. High-quality blastocysts (HBs) were defined as blastocysts ≥3 BB (AA, AB, BA, BB). Low-quality blastocysts (LBs) were defined as vitrified blastocysts, excluding those HBs.

Blastocyst vitrification and warming procedures. The procedure was always performed using one blastocyst for each straw. An artificial shrinkage (AS), using a laser pulse was performed before vitrification. The blastocyst was then moved at room temperature (22-25˚C) to Kitazato (Japan) equilibration solution (ES). After 6-8 min, the blastocyst was quickly washed in vitrification solution (VS) for 45-60 sec and transferred onto the straw (Kitazato Japan) using a micropipette and immersed vertically into liquid nitrogen.

A Kitazato Thaw Kit Kitazato) was used for warming. The carrier containing the embryo was removed from the straw and placed quickly into the dish containing the thawing medium (thawing solution) preheated at 37˚C. The blastocysts immediately fell from the device and could be easily identified in the medium. After 1 min, the blastocysts were transferred to the DS medium (dilution solution) for 3 min at room temperature (22-25˚C). In the last two step, the blastocysts were placed for 5 min, in the WS1 medium and WS2 (washing solution). The embryo was then returned to G-2 medium for culture until transfer. At this stage, an assessment was performed on an inverted microscope to establish if the embryo survived based on morphological integrity of the ICM and trophectoderm. After 1 or 2 h of culture, the embryo was reassessed again and often the re-expansion of the blastocoel was reported; this indicated that the embryo physiologically survived the warming procedure. Embryo transfer was normally performed within 2 or 3 h. All programmed warmed cycles, both at D5 and D6, were transferred in D5 endometrium.

If the patient had both D5 and D6 blastocysts, the best quality embryo was warmed first. If blastocyst quality was the same, the D5 blastocyst was given priority to transfer. If the embryo did not survive, another embryo was warmed if the patient had any in storage, otherwise the transfer was canceled. Some patient blastocysts were not thawed, did not survived, or two blastocysts transferred were not included in this study.

Endometrial programming and observational indicators. All vitrified-warmed cycles of endometrium preparation were natural cycle (NC) or artificial cycle [hormone replacement therapy (HRT)] based on the implantation programs. NC was applicable for patients with a regular menstrual cycle. Follicular development was monitored using B ultrasound on days 8-10 of menstruation. The follicular and endometrial development conditions were assessed and combined with the estradiol (E_2) and luteinizing hormone (LH) levels to confirm the ovulation time. Embryo transfer was performed on D5 of ovulation. HRT was applicable for patients with an irregular menstrual cycle, ovulation disorder, or poor endometrial and follicular development in NC. Starting from days 2-3 of menstruation, 2-6 mg/day of estradiol valerate (Progynova, Bayer) was administered, and the endometrial thickness and serum E_2 levels were monitored using B ultrasound. When the endometrial thickness was at least 8 mm, progesterone 60 mg/day was additionally administered. Embryo transfer was performed on day 6 of progesterone injection. All warmed blastocysts, both vitrified on D5 or D6 were replaced in the D5 endometrium. All embryo transfers were performed using transabdominal ultrasound guidance.

Observation of the gestational sac and fetal heart by B ultrasound at 35 days after implantation was diagnosed as clinical pregnancy. The implantation rate was defined as the ratio between the number of gestational sacs and fetal heart observed under B ultrasound and the number of transferred blastocysts. Implantation rates, pregnancy rates, and twinning of D5/D6 SVBT were analyzed.

Statistical analyses. Statistical analyses were performed using SPSS 19.0 statistical software (SPSS Inc.). The data are presented as the mean ± standard deviation (SD). The mean values of two groups were compared using the independent samples t-test. Percentages were compared using the χ^2 test and $P<0.05$ was considered statistically significant.

Results

D5/D6 HB blastocyst rate in 1,560 fresh IVF/ICSI cycles. The total HB rate was 50.5% (2,688/5,328) for which IVF was higher than ICSI (52.7% vs. 42.6%; $P<0.05$). The D5 HB rate was much higher than the D6 HB rate

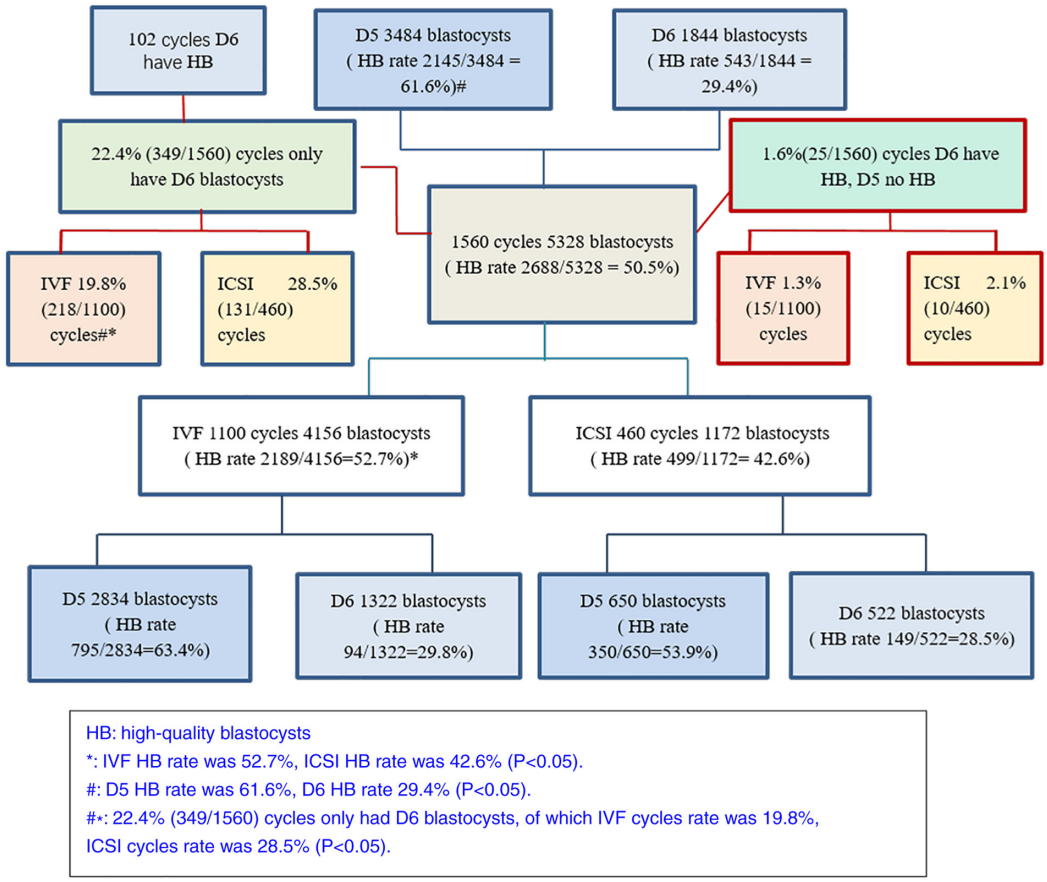

Figure 1. Comparison of the HB rate in D5 and D6 blastocysts of fresh IVF/ICSI cycles. HB, high-quality blastocyst; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection.

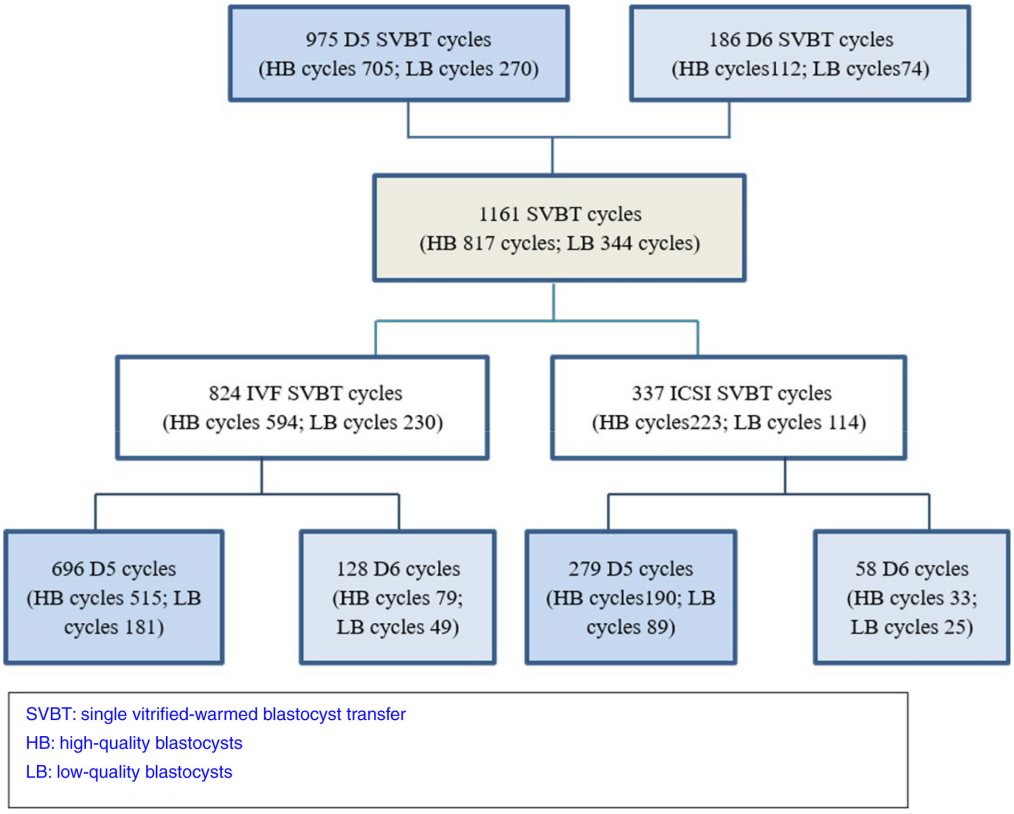

Figure 2. Clinical pregnancy rate of HB/LB SVBT on D5 and D6 in IVF/ICSI. HB, high-quality blastocyst; LB, low-quality blastocyst; SVBT, single vitrified-warmed blastocyst transfer; D5, day 5; D6, day 6; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection.

Table I. HB/LB pregnancy data between the day 5 (D5) and day 6 (D6) groups.

Variables	Groups	Day 5	Day 6	χ^2	P-value
No. of patients		975	186		
No. of high-quality blastocyst (HBs)		705	112		
No. of low-quality blastocysts (LBs)		270	74		
Clinical pregnancy rate (%)	Total	57.4 (560/975)	46.2 (86/186)[a]	7.94	0.0045
	HBs	60 (426/705)	54.5 (61/112)	1.43	0.2320
	LBs	49.6 (134/270)	33.8 (25/74)[a]	5.87	0.0150
Implantation rate (%)	Total	58.9 (574/975)	47.3 (88/186)[a]	8.52	0.0025
	HBs	62.0 (437/705)	56.3 (63/112)	1.34	0.2470
	LBs	50.7(137/270)	33.8 (25/74)[a]	6.70	0.0100
Multiple pregnancy rate (%)	Total	1.44 (14/975)	1.08 (2/186)	0.149	0.7150
	HBs	1.56 (11/705)	1.78 (2/112)	-	0.6960
	LBs	1.11 (3/270)	(0/74)	-	-
Male rate (%)	Total	54.0 (233/431)	48.4 (31/64)	0.708	0.3500
	HBs	53.9 (181/336)	50.0 (24/48)	0.253	0.6150
	LBs	54.7 (52/95)	43.8 (7/16)	0.664	0.4150

[a]P<0.05, significant difference.

Table II. IVF/ICSI pregnancy data between the day 5 (D5) and day 6 (D6) groups of SVBT.

Variables	Groups	IVF	ICSI	χ^2	P-value
No. of patients		824	337		
No. of D5 blastocysts		696	279		
No. of D6 blastocysts		128	58		
Clinical pregnancy rate (%)	Total	56.3 (464/824)	54.0 (182/337)	0.515	0.470
	D5	58.0 (404/696)	55.9 (156/279)	0.370	0.543
	D6	46.8 (60/128)	44.8 (26/58)	0.067	0.795
Implantation rate (%)	Total	57.0 (473/824)	56.1 (189/337)	0.035	0.860
	D5	59.2 (412/696)	58.1 (162/279)	0.105	0.746
	D6	47.7 (61/128)	46.6 (27/58)	0.020	0.889
Multiple pregnancy rate (%)	Total	1.09 (9/824)	2.08 (7/337)	1.71	0.175
	D5	1.15 (8/696)	2.15 (6/279)	0.792	0.374
	D6	0.78 (1/128)	1.72 (1/58)	-	0.528
Male rate (%)	Total	52.2 (189/362)	56.4 (75/133)	0.68	0.430
	D5	52.8 (167/316)	57.4 (66/115)	0.701	0.403
	D6	47.8 (22/46)	50.0 (9/18)	0.024	0.876

IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection; SVBT, single vitrified-warmed blastocyst transfer.

(61.6% vs. 29.4%; P<0.05). There were 22.4% (349/1560) cycles of only cultured D6 blastocysts, in which IVF was lower than ICSI (19.8% vs. 28.5%; P<0.05) (Fig. 1).

Clinical pregnancy rate of HB/LB SVBT on D5 and D6 in IVF/ICSI. In total, 1,161 SVBT cycles (D5 975 and D6 186) were analyzed (Fig. 2). The mean age of the women in the D5 group and D6 group was not different (31.8 years vs. 31.9 years). The clinical pregnancy rate and implantation rate in the D5 group were significantly higher than these rates in the D6 group (57.4 vs. 46.2%, 58.9 vs. 47.3%; P<0.05). However, the clinical pregnancy rate and implantation rate of D5 HB were not significantly different from those of D6 HB (60 vs. 54.5%, 62 vs. 56.3%; P>0.05). The clinical pregnancy rate and implantation rate of D5 LB were higher than those of D6 LB (49.6 vs. 33.8%, 50.7 vs. 33.8%; P<0.05) (Table I). The clinical pregnancy rate and implantation rate were similar in IVF and ICSI groups (56.3 vs. 54.0%, 57.0 vs. 56.1%; P>0.05) (Table II). The multiple pregnancy rate was similar in the D5/D6 groups, and ICSI was higher than IVF, but not statistically significant (1.44 vs. 1.08%, 2.08 vs. 1.09%; P>0.05). The D5 male rate was higher than the D6 male rate (54.0

vs. 48.4%), but was not statistically significant (Table I). The male rate was similar in the IVF/ICSI groups (52.2 vs. 56.4%; P>0.05) (Table II).

Discussion

Extending embryo culture to the blastocyst stage has become a routine in many *in vitro* fertilization (IVF) laboratories. The most widely used grading system is that originally proposed by Gardner and Schoolcraft (13). Although the system does not cover all aspects of blastocyst morphology it has been very effective in classifying the appearance and compactness of the inner cell mass (ICM), the cohesiveness and number of trophectoderm (TE) and degree of expansion of the blastocoel cavity.

Whether there are differences in the pregnancy outcomes of blastocysts cryopreserved during different developmental stages remains under debate because the results among studies are inconsistent. A meta-analysis of clinical outcomes showed that in day 5 (D5) vs. day 6 (D6) blastocyst transfers, clinical pregnancy rate and live birth rates were significantly higher following D5 compared to D6 blastocyst transfers (14). Therefore, ART practitioners should preferably transfer D5 rather than D6 blastocysts in both fresh and frozen cycles (14). Single embryo transfers of D6 vitrified/warmed blastocysts were found to result in a lower implantation and clinical pregnancy rate compared to D5 embryos (15). The effect of delayed blastulation may be responsible for implantation failures and negatively affects outcomes (4). However, in their studies, Behr *et al* (16) and El-Toukhy *et al* (9) did not observe a significant difference in the implantation and pregnancy rates between D5 and D6 blastocysts.

In addition, blastocyst grade plays an important role in pregnancy outcomes. Blastocysts with trophectoderm grades A, B, and C were found to have euploidy rates of 71.43, 60.00 and 19.67%, respectively (P<0.05) (17). Yang *et al* (11) reported that high-quality D6 blastocysts in vitrified-warmed cycles had similar developmental potential and pregnancy outcomes compared to those of high-quality D5 blastocysts, while Irani *et al* (18) observed that embryos reaching good-quality blastocysts on day 5 yielded significantly higher implantation rate (77.7% vs. 58.7%) compared with those reaching similar quality blastocysts on day 6. Similarly, D5 average-quality embryos conveyed a significantly higher implantation rate compared with D6 embryos of the same quality (64.4% vs. 53.4%) (18).

In previous research, patients who underwent single vitrified-warmed blastocyst transfer (SVBT) cycles were able to obtain optimal pregnancy outcomes, especially in the <35 year age group (19), while those older than 35 years may have a higher probability of pregnancy failure due to chromosomal abnormalities, age or other factors (20). This is why our research selected patients age <35 years and without biopsy. The multiple pregnancy rate was 1.08% (0.98% for D5 vs. 1.3% for D6) (15), which was similar to our results.

While the previous studies focused on warming embryo of D5/D6 frozen embryo transfer (FET) cycles, the present study was the first to consider the high-quality blastocyst (HB) rate in fresh *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) cycles, and the rate of D6 had blastocysts whereas D5 had none. The present results showed that the D5 HB rate was twice higher than the D6 HB rate in fresh cycles (61.6% vs. 29.4%; P<0.05), and this was probably the reason why the clinical pregnancy rate and implantation rate in the D5 group were significantly higher than these rates in the D6 group (57.4% vs. 46.2%, 58.9% vs. 47.3%; P<0.05).

From this research, we know that in IVF, the cultured blastocysts and HBs per cycle were more than these parameters in ICSI, and cultured blastocysts in IVF were earlier than ICSI. We concluded that the fertilization method directly influenced HB and blastocyst development rates. Therefore, the IVF/ICSI ratio needs to be considered when analyzing D5/D6 SVBT. In the present SVBT study, D5 (IVF 71.4% and ICSI 28.6%) cycles and D6 (IVF 68.8% and ICSI 31.2%) IVF/ICSI ratios were not significantly difference. Speyer *et al* (21) also showed that IVF-derived embryos developed to the blastocyst stage at a significantly faster rate than ICSI-derived embryos. A previous study using time-lapse showed the different developmental time between IVF and ICSI embryos. During the early cleavage stages there was a statistically significant delay (+1.5 to +1.1 h) among the IVF-fertilized embryos, and at the blastocyst stage IVF-fertilized embryos showed faster development (22). IVF/ICSI sibling oocyte split design demonstrated a higher-quality blastulation rate in the IVF group compared to the ICSI group when calculated per 2PN, but not per oocyte allocated to each insemination procedure (23).

Most patients prefer to use D5 HB in their first FET cycle, and finally choose D6 blastocysts when none of the thawed D5 blastocysts have resulted in successful pregnancy. Therefore, the inclusion criteria were patients who were in their first fresh cycle without fresh embryo transfer and who were undergoing their first SVBT cycle.

In conclusion, following control of patient age, transfer frequency, and endometrium on day 5, it is not the development stage (D5/D6) but the transfer blastocyst quality that plays an important role in achieving the optimal pregnancy outcomes. The D5 HB rate was found to be 2-times higher than D6, and the IVF HB rate was also higher than ICSI, which may be the reason for the current debate in the literature regarding the pregnancy outcomes of D5/D6 SVBT.

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Availability of data and materials

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

Authors' contributions

YJ conceived the study and wrote the paper. YJ and GS performed the experiments and analyzed the data. YJ and XHZ contributed to design and conception. SBM and XHW

contributed to acquisition and interpretation of data. GS, XHZ, SBM and XHW confirmed the authenticity of all of the data. XHW supervised the study. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The Fourth Hospital of Shijiazhuang Ethics Committee approved (approval no. 20170063; approval date, January 5, 2017) this study. The procedures used in this study adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from all individual participants included in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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