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GYNECOLOGIC ENDOCRINOLOGY AND REPRODUCTIVE MEDICINE

Prioritized single vitrified blastocyst to be warmed between grades 3 or 4 blastocyst on day 5 transfer cycles

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Abstract

Purpose Selecting the optimal blastocyst to implant during cryopreservation and warming is critial for in vitro fertilization success. Therefore, the aim of this study was to explore which blastocyst should be prioritized to be thawed when facing a single vitrified blastocyst on day 5 transfer.

Methods A retrospective study including 1,976 single vitrified-warmed blastocyst transfer cycles was conducted from January 2016 to December 2020.

Results We found that grade 4 vitrified blastocyst had a higher clinical pregnancy (60.64% vs. 49.48%, $P < 0.001$) and live birth rates (50.12% vs 39.59%, $P < 0.001$) than the grade 3 vitrified blastocyst. However, no statistical difference was found between groups in miscarriage rate, birth weight, or gestational age. Besides, the grade 4 vitrified–thawed blastocyst had significant potential to develop into grade 6 blastocyst after further culturing for 16 h (73.68% vs. 48.60%, $P < 0.001$). The grade 6 transferred blastocyst was markedly higher in both clinical pregnancy rate (61.88% vs. 51.53%, $P < 0.001$) and live birth rate (50.91% vs. 40.46%, $P < 0.001$) compared to grade 5 transferred blastocyst.

Conclusions Grade 4 vitrified blastocyst is recommended when facing single vitrified blastocyst on day 5 transfer. More importantly, the "embryonic escape hypothesis" was firstly proposed to reveal the findings.

Keywords Single blastocyst transfer · Embryo quality · Expansion · Pregnancy rate · Embryonic escape hypothesis · Vitrified–warmed

What does this study add to the clinical work

The "embryonic escape hypothesis" was firstly proposed to reveal the findings that grade 4 vitrified blastocyst is recommended when facing single vitrified blastocyst on day 5 transfer. It's worthwhile for physicians to provide advice to patients during FET treatment.

Background

With the rapid development of assisted reproductive technology, the ultimate target has shifted from achieving successful pregnancy to having a single, healthy, and full-term infant. Currently, single embryo transfer (SET) is considered the optimum solution to achieve this aim [1]. A mass of literature reported that SET dramatically avoids the complication of multiple pregnancies for mothers and neonates, including gestational diabetes mellitus and hypertension, maternal mortality, prematurity, and intrauterine growth restriction [2, 3]. Meanwhile, SET obtains favorable clinical pregnancy and live birth rates [4, 5].

Over the last few decades, the single vitrified blastocyst transfer has become increasingly popular and widely used worldwide due to the technique refinements in embryo cryopreservation [6] and the low aneuploidy rate in the blastocyst. A previous study suggested that single vitrified blastocyst transfer is better than single fresh blastocyst transfer to yield singleton live birth [7]. Additionally, single-vitrified blastocyst transfer is confirmed superior to double-vitrified

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cleavage embryo transfer, not only in clinical pregnancy rate but also in implantation and ongoing pregnancy rates [8, 9].

Therefore, selecting the optimal implantation potential blastocyst is essential for cryopreservation and warming. Gardner and Schoolcraft morphological scoring [10], which is a simple and noninvasive method, is most commonly used by embryologists to assess blastocyst quality. It includes three morphological parameters, including the degree of expansion and the development of the inner cell mass (ICM) and trophectoderm (TE). To date, many investigations revealed that these three features are all associated with blastocyst quality and could predict pregnancy outcomes [11–13]. The most frequently to be vitrified are grades 3 or 4 blastocysts among embryo cryopreservation on day 5 [14–16]. However, to our best knowledge, no study has explored the blastocyst that should be prioritized to be thawed when facing a single vitrified blastocyst on day 5 transfer.

Therefore, this retrospective study aimed to compare the clinical outcomes according to blastocyst expansion (grade 3 or grade 4) and ICM and TE quality (good or poor) to provide reasonable proposals for clinicians and patients.

Materials and methods

Study design

The study was conducted following the guidelines established by the Ethics Committee of Nanjing Maternity and Child Health Care Hospital (NO: NJFY-2020KY-051). This retrospective study compared the clinical outcomes after vitrified blastocyst transfer from January 2016 to December 2020 in the reproductive center of the hospital. Only single vitrified blastocyst on day 5 transfer cycles were included. Cycles with preimplantation genetic testing were excluded.

According to the stage of expansion and quality of vitrified blastocyst, namely, the grades of ICM and TE cells, six study groups were classified: (1) single grade 3 good-quality blastocyst, (2) single grade 3 poor-quality blastocyst, (3) single grade 4 good-quality blastocyst, (4) single grade 4 poor-quality blastocyst, (5) single stage ≥ 5 good-quality blastocyst, and (6) single stage ≥ 5 poor-quality blastocyst, all on day 5.

Ovarian stimulation

The flexible gonadotropin-releasing hormone (GnRH) antagonist protocol was applied to participants. The follicle-stimulating hormone (Gonal-F, rFSH, Merck Serono, Germany), varying from 150 to 225 IU/day, was used from the third day of the menstrual cycle. The ultrasound scan and sex hormone levels (estradiol, FSH, luteinizing hormone, and progesterone) were adopted to monitor follicular growth. Meanwhile, the gonadotropin (Gn) dose was adjusted according to the above results. After Gn was applied for 5 ~ 6 days, 0.25 ~ 0.5 mg/d of GnRH antagonist (Cetrotide, Merck Serono, Germany) was added until the day of human chorionic gonadotrophin (HCG). A 250-μg human chorionic gonadotrophin (rHCG, Merck Serono, Germany) injection was given to stimulate final oocyte maturation when three follicles ≥ 17 mm are observed, then oocyte retrieval was conducted after 36 h.

Embryo culture and evaluation

Conventional insemination IVF or intracytoplasmic sperm injection (ICSI) was executed based on semen parameters. Fertilization was assessed after 16 ~ 18 h of insemination or injection. Subsequently, the embryos were cultured and graded until day 5. The quality of blastocysts was evaluated by the same embryologist, according to Gardner and Schoolcraft's criteria [10]. The embryos were good-quality blastocysts when the ICM and TE scores were both above B (AA, AB, BA, and BB). On the contrary, the embryos were poor-quality blastocysts when the ICM or TE scores were below B (AC, BC, CA, CB, and CC). The "overall" in Tables 2, 3 and 7 referred to the total blastocysts including good-quality blastocysts and the poor-quality blastocysts. The oocytes and embryos were cultured in a sequential medium (IVF, G1 and G2, Vitrolife, Sweden) with an environment of 37 °C, 6% carbon dioxide, 5% oxygen, 89% nitrogen, and saturated humidity (Astec, Japan).

vitrification and warming of embryos

Blastocysts were vitrified using the vitrification method. First, the blastocyst was subjected to laser pulse artificial shrinking (AS) under the laser beam (ZILOS-tk system, Hamilton Thorne Instruments Biosciences, Beverly, MA, USA). The detailed protocols of AS were according to those previously described [17]. Briefly, the shot was placed in the opposite of ICM cells and 200 ms laser pulse was used. Then, the blastocyst was transferred into an equilibration solution of a vitrification media kit (Kitazato, Shizuoka, Japan) for 12 min, including 7.5% ethylene glycol and 7.5% dimethylsulphoxide (DMSO). Subsequently, blastocysts were soaked in a vitrification solution of a vitrification media kit (Kitazato, Shizuoka, Japan) for 1 min, containing 15% of ethylene glycol, 15% of DMSO, and 17% of sucrose. Finally, blastocysts were vitrified at the top of the Cryotop device and quickly placed into liquid nitrogen.

For warming, the Cryotop was immediately submerged into a warming solution of warming media kit (Kitazato, Shizuoka, Japan) for 1 min, including 34% of sucrose. Then, blastocysts were transferred to a diluent solution of warming

media kit (Kitazato, Shizuoka, Japan) for 3 min, consisting of 17% of sucrose. Afterward, the blastocysts were cleaned in a washing solution of a warming media kit (Kitazato, Shizuoka, Japan) for 5 min, without sucrose. Subsequently, the blastocyst was transferred into a culture medium and subjected to assisted hatching (AH) to thin out one-third of zona pellucida. The shot was placed at a distance from the ICM cells by using a laser beam with a 200 ms laser pulse (ZILOS-tk system, Hamilton Thorne Instruments Biosciences, Beverly, MA, USA). The vitrified blastocyst was warmed in the afternoon of day 5 after progesterone administration and cultured for an additional 16 h, then, the blastocysts were re-evaluated and transferred. The vitrification, warming solutions, and Cryotop were taken from Kitazato Corporation (Kitazato, Shizuoka, Japan).

Survival assessment of vitrified-warmed blastocyst

Following 1–3 h of culture after warming, if the blastocoele re-expand completely or partially, the blastocyst survives. If culturing for overnight, the blastocoele is still not re-expand, and the blastocyst is dead.

Endometrial preparation and vitrified embryo transfer

Participants were treated under a natural cycle (NC) or artificial cycle (AC) or ovulation induction cycle (OIC)according to the menstrual cycles.

A serial transvaginal ultrasonography was used from day 10 to 12 of menstrual cycles to monitor the endometrial thickness and follicular growth for natural endometrial preparation. Participants were injected with 10,000 IU of hCG when the dominant follicle is > 18 mm, the endometrial thickness was ≥ 7 mm, and the progesterone level was ≤ 1.5 ng/ml. An ultrasound was used to confirm ovulation approximately 1–2 days later. Oral dydrogesterone (30 mg/d, Abbott Biologicals B.V., Netherlands) was started at ovulation confirmation (P + 0) until 2 weeks after vitrified embryo transfer (FET).

Participants received oral estrogen (estradiol valerate, Progynova, Bayer, France) at initiation dosage (4–6 mg/d) from day 2–8 of the menstrual cycle for artificial endometrial preparation. The dosage was changed to 8–10 mg/d according to the serum estradiol (E2) and endometrial thickness, thereafter. AC was further divided into two subgroups based on the GnRH agonist (triptorelin acetate, Diphereline, IPSEN, France), including the hormonal replacement cycle and down-regulation + hormonal replacement cycle. The GnRH agonist was given during the follicular period (day 2–4). The down-regulation was confirmed when the expected hormone and endometrial thickness (E2 of > 30 pg/ml), luteinizing hormone and FSH levels (< 5 IU/L), and endometrial thickness (< 5 mm) were achieved. Then, oral estrogen was received at 4–6 mg/d. Vaginal progesterone (90 mg/d, Crinone, Merck Serono, Germany) and dydrogesterone (10 mg/d) were commenced (P + 0) when the endometrial thickness of ≥ 7 mm and serum E2 level of ≤ 200 pg/ml were achieved. Oral dydrogesterone (30 mg/d, Abbott Biologicals B.V., Netherlands) was given from ovulation until 12 weeks after gestation.

HMG (75 U/d, Lizhu, China) was intramuscularly injected starting from days 3–5 of the menstrual cycle for endometrial preparation for the ovulation induction cycle. Subsequently, the dosage was adjusted according to the follicular size and hormonal levels. HCG of 10,000 IU was intramuscularly injected to stimulate ovulation in endometrial thickness of ≥ 7 mm and follicular size of > 18 mm. Oral dydrogesterone (30 mg/d, Abbott Biologicals B.V., Netherlands) was given from ovulation until 12 weeks after gestation.

Blastocysts transfer was conducted on the fifth day after ovulation (for the NC and OIC regimen) or on the sixth day of progesterone exposure (P + 6) (for AC regimen) with a Wallace catheter (Smith Medical International Ltd. UK) and an abdominal ultrasound scan. The serum β-HCG level was measured after 14 days.

Clinical outcomes

The primary study outcomes were the clinical pregnancy rate. The secondary outcomes were live birth rate, miscarriage rate, and neonatal outcomes, including gestational age, birth weight, and neonatal gender. The clinical pregnancy was based on gestational sac detection by ultrasound after 6 weeks of pregnancy. Live birth was termed as a fetus born alive after 28 weeks of gestation. Miscarriage was considered as the loss of a spontaneous clinical pregnancy before 28 weeks of gestation.

Statistical analysis

The comparisons of baseline characteristics and neonatal outcomes were presented as the mean ± standard deviation (SD) for continuous data and percentage for categorical data. Pearson's chi-square test was applied for categorical variables and independent samples *t* test or the Mann–Whitney U test (when the data were not normally distributed) was used for the descriptive variables between groups. As for that, more than one cycle from the same couples were taken into, the association between repeated embryo transfer cycles by the same couples might affect the statistical analysis. So the generalized estimated equation regression model was conducted to identify the relation between embryo quality, development stage of embryo and clinical outcomes. The outcomes were described as unadjusted and adjusted *P*

Table 1 Female demographic and treatment characteristics of single vitrified-thawed transfer cycles

	Vitrified grade 3 blastocysts		Vitrified grade 4 blastocysts		Vitrified grade ≥ 5 blastocysts	
	Good quality	Poor quality	Good quality	Poor quality	Good quality	Poor quality
Number of FET cycles, *n* (%)	386 (19.53)	291 (14.73)	1044 (52.83)	229 (11.59)	23 (1.16)	3 (0.16)
Female age(years), mean ± SD	31.30 ± 4.50	31.53 ± 4.84	30.72 ± 3.96	31.83 ± 4.71	30.91 ± 3.70	32 ± 3.46
Female BMI (kg/m^2), mean ± SD	22.41 ± 3.41	22.46 ± 3.28	22.36 ± 3.36	22.78 ± 3.28	22.32 ± 3.45	21.25 ± 1.38
Type of infertility, *n* (%)						
Primary infertility	204 (52.85)	128 (43.99)	484 (46.36)	97 (42.36)	8 (34.78)	–
Second infertility	182 (47.15)	163 (56.01)	560 (53.64)	132 (57.64)	15 (65.22)	3 (100.00)
FET cycles rank						
1	234 (60.62)	99 (34.02)	814 (77.97)	128 (55.90)	19 (82.61)	2 (66.67)
2	109 (28.24)	123 (42.27)	180 (17.24)	81 (35.37)	4 (17.39)	–
≥ 3	43 (11.14)	69 (23.71)	50 (4.79)	20 (8.73)	–	1 (33.33)
Endometrial preparation program, *n* (%)						
Natural cycle	69 (17.88)	37 (12.71)	154 (14.75)	37 (16.16)	3 (13.04)	–
Hormonal replacement cycle	198 (51.30)	181 (62.20)	537 (51.44)	124 (54.15)	14 (60.87)	3 (100.00)
Down regulation + hormonal replacement cycle	85 (22.02)	54 (18.56)	260 (24.90)	48 (20.96)	5 (21.74)	–
Ovulation induction cycle	34 (8.81)	19 (6.53)	93 (8.91)	20 (8.73)	1 (4.35)	–
Method of fertilization						
IVF	290 (75.12)	207 (71.34)	815 (78.07)	170 (74.24)	17 (73.91)	1 (33.33)
ICSI	96 (24.88)	84 (28.66)	229 (11.93)	59 (25.76)	6 (16.09)	2 (66.67)

value, odds ratiois (OR) and 95% confidence intervals (CIs). $P < 0.05$ was considered to be statistically significant. Statistical analyses were conducted using the Stata 11.0 statistical software package (Stata Corp, LP).

Results

Study population

This study retrospectively analyzed 1,976 single vitrified–thawed embryos on day 5 transfer cycles. Of these, 386(19.53%) cycles have grade 3 good-quality vitrified blastocyst, 291 (14.73%) cycles have grade 3 poor-quality vitrified blastocyst, 1,044 (52.83%) cycles have grade 4 good-quality vitrified blastocyst, 229 (11.59%) cycles have grade 4 poor-quality vitrified blastocyst, 23 (1.16%) cycles have stage ≥ 5 good-quality vitrified blastocyst, and 3(0.16%) cycles have stage ≥ 5 poor-quality vitrified blastocyst. The patient's baseline characteristics were described, as summarized in Table 1. The body mass index (BMI), the proportion of NC and the methods of fertilization were comparable among the groups (grade 3 good-quality vs. grade 3 poor-quality; grade 4 good-quality vs. grade 4 poor-quality; grade 3 good-quality vs. grade 4 good-quality; grade 3 poor-quality vs. grade 4 poor-quality; $P > 0.05$). However, the grade 4 good-quality group had statistically younger maternal age than the grade 4 poor-quality group (30.72 ± 3.96 vs. 31.83 ± 4.71, $P = 0.003$), without difference between other groups in terms of maternal age. Additionally, the grade 3 good-quality group had a higher proportion of primary infertility than the grade 3 poor-quality (52.85% vs. 43.99%, $P = 0.022$), as well as grade 4 good-quality (52.85% vs. 46.36%, $P = 0.029$), without difference between other groups. Moreover, significant differences were observed in the proportion of patients who underwent their first FET between all groups ($P < 0.001$).

We conducted a two-stage association study, firstly, the relationship between vitrified blastocyst quality and clinical outcomes, secondly, the association between pre-transferred blastocyst quality and IVF success rate.

Analysis of vitrified embryo quality and clinical treatment outcomes

First, we calculated the clinical outcomes grouped by different stages of the vitrified blastocyst, including clinical pregnancy, live birth, and miscarriage rates, and the results were presented in Table 2 and Fig. 1& 2.

As listed in Table 2 and Fig. 1. A, the cycles of grade 4 vitrified blastocysts were approximately double that of grade 3 vitrified blastocysts on day 5, and the good-quality blastocyst rate was significantly higher in grade 4 blastocysts than in grade 3 blastocysts on day 5(57.02% vs. 82.01%, $P < 0.001$). Tables 2, 3 and Fig. 1. B, C demonstrated that the grade 3 good-quality group had a higher clinical pregnancy

Table 2 Clinical treatment outcomes of single vitrified-warmed transfer cycles

	Vitrified grade 3 blastocysts			Vitrified grade 4 blastocysts			Vitrified grade ≥ 5 blastocysts		
	Good quality	Poor quality	Overall	Good quality	Poor quality	Overall	Good quality	Poor quality	Overall
FET cycles *n* (%)	386 (57.02%)[a]	291 (42.98%)	677 (100%)	1044 (82.01%)[a]	229(17.99%)	1273 (100%)	23 (88.46%)	3 (11.54%)	26 (100%)
Clinical pregnancy	56.74%	39.86%	49.48%	63.03%	49.78%	60.64%	52.17%	33.33%	50.00%
Live birth	47.67%	28.87%	39.59%	52.87%	37.55%	50.12%	43.48%	33.33%	42.3%
Miscarriage	15.07%	26.72%	19.10%	15.35%	21.93%	16.32%	8.33%	–	7.69%

[a]Compared to grade 3 blastocyst, grade 4 was significantly higher in the good-quality rate, $P < 0.001$. Overall refers to the blastocysts including good quality and poor quality

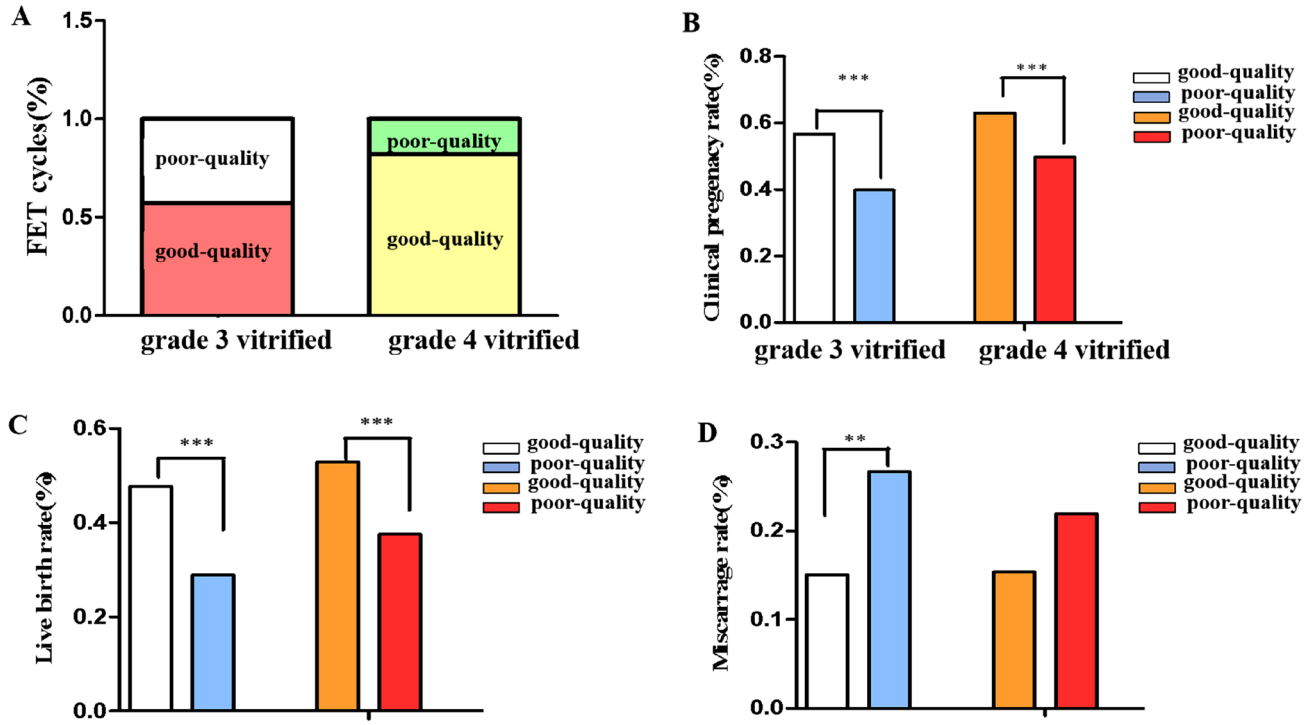

Fig. 1 Comparison of clinical outcomes between good-quality and poor-quality in grade 3 & 4, respectively. **A** presented the proportion of good-quality and poor-quality in grade 3 & 4 vitrified blastocysts. **B** compared the clinical pregnancy rate of good quality and poor quality in grade 3 & 4, respectively. **C** listed the live birth rates of good quality and poor quality in grade 3 & 4, respectively. **D** showed the miscarriage rate of good quality and poor quality in grade 3 & 4, respectively

rate (56.74% vs. 39.86%, $P < 0.001$) and a higher live birth rate (47.67% vs. 28.87%, $P < 0.001$) than the grade 3 poor-quality group. And Tables 2, 3 and Fig. 1. D showed that the grade 3 good-quality group had a lower miscarriage rate (15.07% vs. 26.72%, $P = 0.009$). The coherence of the changing trend was the same in the grade 4 good-quality group when compared with the grade 4 poor-quality group in these three rates (Tables 2, 3 and Fig. 1).

Interestingly, we noticed that grade 4 good-quality group had higher clinical pregnancy rate (63.03% vs. 56.74%, $P = 0.030$) and live birth rate (52.87% vs. 47.67%, $P = 0.080$) (Tables 2, 3 and Fig. 2A, B) than grade 3 good-quality group. However, no difference was found in miscarriage rate between the two groups (15.35% vs. 15.07%, $P = 0.920$) (Tables 2, 3 and Fig. 2C). Besides, when comparing the grade 4 poor-quality group and grade 3 poor-quality group, we found similar results in Tables 2, 3 and Fig. 2A–C.

Moreover, we compared the grade 3 overall group with the grade 4 overall group and revealed that the grade 4 overall group had a higher clinical pregnancy (60.64% vs. 49.48%, $P < 0.001$) and live birth rates (50.12% vs 39.59%, $P < 0.001$) than the grade 3 overall group. However, no significant difference was found in the miscarriage rate between

Table 3 Generalized estimated equation model explored the association of embryo quality, embryo development stage and clinical outcomes

	Clinical pregnancy				Live birth				Miscarriage			
Good-quality vs poor-quality[a]	*P*	OR (95% CI)	Adjusted *P*	Adjusted OR (95% CI)	*P*	OR (95% CI)	Adjusted *P*	Adjusted OR (95% CI)	*P*	OR (95% CI)	Adjusted *P*	Adjusted OR (95% CI)
Grade 3 blastocysts	**< 0.001**	**1.98 (1.45 2.69)**	**0.002**	**1.70 (1.22 2.37)**	**< 0.001**	**2.24 (1.63 3.10)**	**< 0.001**	**2.02 (1.43 2.85)**	**0.009**	**0.49 (0.28 0.84)**	**0.014**	**0.47 (0.26 0.86)**
Grade 4 blastocysts	**< 0.001**	**1.72 (1.29 2.29)**	**0.023**	**1.41 (1.05 1.91)**	**< 0.001**	**1.87 (1.39 2.50)**	**0.001**	**1.66 (1.23 2.25)**	0.079	1.55 (0.91 2.58)	0.100	0.66 (0.40 1.08)
Grade 3 vs grade 4[b]												
Good-quality blastocysts	**0.030**	**0.77 (0.61 0.98)**	0.432	0.90 (0.69 1.17)	0.080	0.81 (0.64 1.03)	0.15	0.84 (0.66 1.07)	0.920	0.98 (0.62 1.52)	0.89	1.03 (0.66 1.60)
Poor-quality blastocysts	**0.024**	**0.67 (0.47 0.95)**	0.187	0.78 (0.54 1.13)	**0.036**	**0.67 (0.47 0.97)**	0.21	0.78 (0.53 1.15)	0.397	1.30 (0.71 2.37)	0.60	1.29 (0.62 2.33)
Overall												
Grade 3 vs grade 4 blastocysts[c]	**< 0.001**	**0.64 (0.53 0.77)**	**0.031**	**0.79 (0.65 0.98)**	**< 0.001**	**0.65 (0.54 0.79)**	**0.002**	**0.73 (0.60 0.89)**	0.259	1.21 (0.85 1.71)	0.25	1.23 (0.87 1.74)

Bold values indicate statistical significance

[a]Good-quality was the reference

[b]Grade 3 was the reference

[c]Grade 4 was the reference

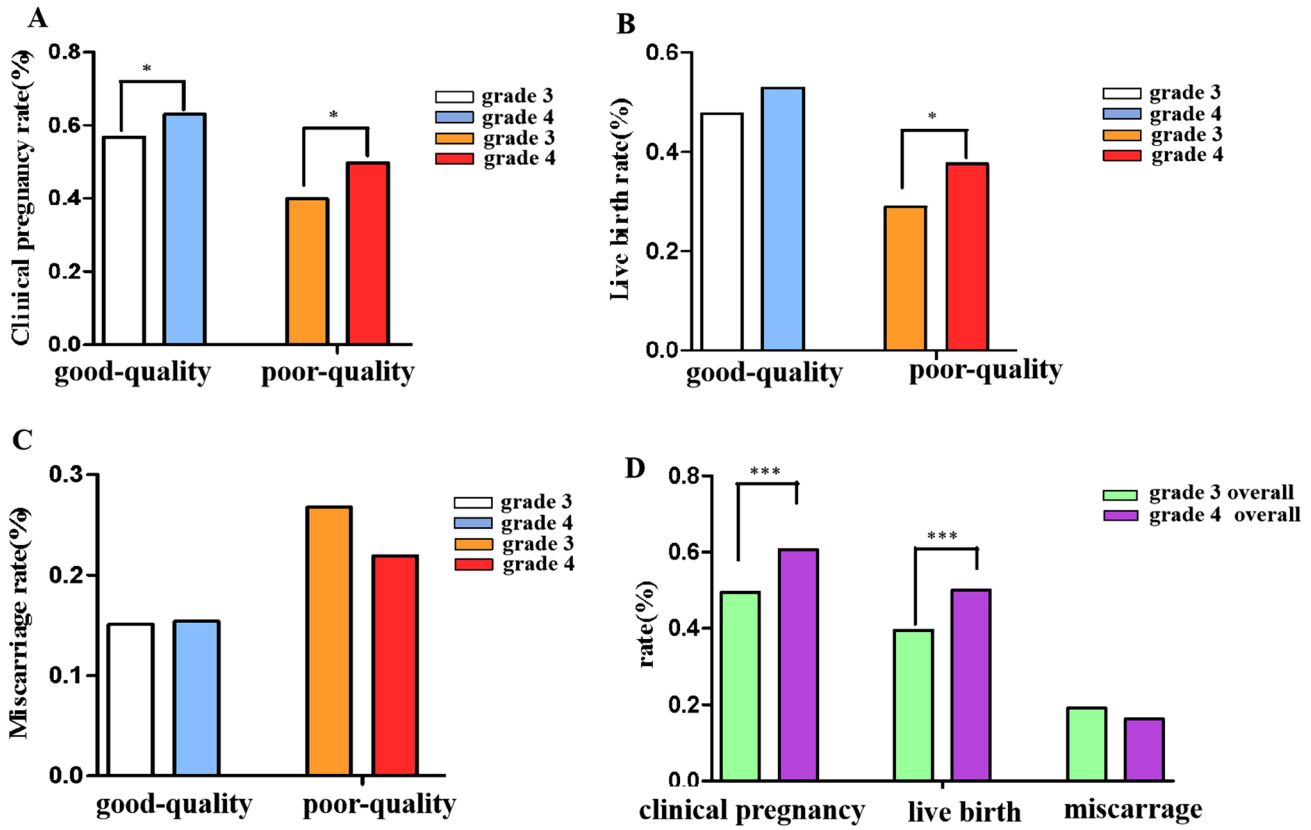

Fig. 2 Comparison of clinical outcomes between grade 3 and 4 vitrified blastocysts in good-quality and poor-quality, respectively. **A** compared the clinical pregnancy rate of grade 3 and 4 vitrified blastocysts in good-quality & poor-quality, respectively. **B** listed the live birth rate of grade 3 and 4 vitrified blastocysts in good-quality & poor-quality, respectively. **C** showed the miscarriage rate of grade 3 and 4 vitrified blastocysts in good-quality & poor-quality, respectively. **D** comparison of clinical outcomes between grade 3 and 4 overall vitrified blastocysts

them (19.10% vs. 16.32%, $P = 0.259$). The results were shown in Tables 2, 3 and Fig. 2D.

Increased OR of 70% in clinical pregnancy rate (adjust OR = 1.70, 95% CI 1.22–2.37), 102% in live birth rate (adjust OR = 2.02, 95% CI 1.43–2.85), and decreased 53% in miscarriage rate (adjust OR = 0.47, 95% CI 0.26–0.86) were found in grade 3 poor-quality vitrified blastocyst group compared with grade 3 good-quality vitrified blastocyst group after controlling for female age, female BMI, type of infertility, FET cycles rank, and endometrial preparation program. A similar trend was found in grade 4 poor-quality group compared with the grade 4 good-quality group, and in grade 3 overall group compared with grade 4 group. However, no difference was found between the grade 3 good-quality group and the grade 4 good-quality group, in clinical pregnancy, live birth, or miscarriage rates. Besides, no difference was found between the grade 3 poor-quality group and the grade 4 poor-quality group in clinical outcomes. The detailed results of the generalized estimated equation that analyzes factors associated with clinical outcomes were described in Supplementary materials 1–5.

Then, we further explored the neonatal outcomes of single vitrified–warmed blastocyst transferred at different expansion stages and blastocyst quality. Interestingly, we found that 51.61% of grade 3 good-quality blastocyst were found to be male, whereas 62.77% of grade 4 good-quality blastocyst were found to be male. A similar trend was found in poor quality between grade 3 and 4. In summary, the higher grade of blastocyst, the more likely to be male newborn. No significant difference was observed in birth weight or gestational age among all groups, as shown in Table 4 and Supplementary material 6.

Analysis of transferred embryo quality and clinical outcomes

Subsequently, we paid attention to the relationship between the transplanted embryo quality and treatment outcomes. First, we assessed the proportion of transferred embryos at different development stages. After 16 h of culture, 6.06% of grade 3 vitrified blastocyst remained in grade 3, 5.61% of grade 3 vitrified blastocyst developed into grade 4 blastocyst,

Table 4 Neonatal outcomes of single frozen embryo transfer, stratified by embryo quality and expansion stage

	Vitrified grade 3 blastocysts		Vitrified grade 4 blastocysts		Vitrified grade ≥ 5 blastocysts	
	Good quality	Poor quality	Good quality	Poor quality	Good quality	Poor quality
Number of neonate	186	84	564	86	11	1
Newborn gender, *n* (%)						
Male	96 (51.61)	50 (59.52)	354 (62.77)	50 (58.14)	10 (90.91)	1 (100.00)
Female	90 (48.39)	34 (40.48)	210 (37.23)	36 (41.86)	1 (9.09)	–
Gestational age, mean ± SD	37.96 ± 2.10	38.14 ± 1.92	38.04 ± 1.88	38.37 ± 1.34	37.27 ± 2.90	37
< 32 weeks	4 (2.15)	1 (1.19)	9 (1.60)	–	–	–
32–37 weeks	20 (10.75)	9 (10.71)	71 (12.59)	7 (8.14)	2 (18.18)	–
≥ 37 weeks	162 (87.10)	74 (88.10)	484 (85.82)	79 (91.86)	9 (81.82)	1 (100.00)
Birthweight, mean ± SD	3344.73 ± 528.58	3451.55 ± 545.59	3359.67 ± 546.11	3422.33 ± 482.83	3330.91 ± 750.35	3600
< 1500 g	1 (0.54)	–	4 (0.71)	–	–	–
1500–2500 g	10 (5.38)	4 (4.76)	26 (4.61)	3 (3.49)	1 (9.09)	–
2500–4500 g	174 (93.55)	80 (95.24)	526 (93.26)	82 (95.35)	10(90.91)	1 (100.00)
> 4500 g	1(0.54)	–	8 (1.42)	1 (1.16)	–	–

Table 5 The developmental outcomes of vitrified-warmed blastocyst (grade 3/4) after 16 h culture

Transfer, *n* (%)	Vitrified grade 3 blastocysts	Vitrified grade 4 blastocysts
Grade 3 blastocysts	41 (6.06)	–
Grade 4 blastocysts	38 (5.61)	80 (6.28)
Grade 5 blastocysts	269 (39.73)	255 (20.03)
Grade 6 blastocysts	329 (48.60)	938 (73.68)
Total	677	1273

Table 6 The origin of transferred blastocysts (grade 5/6)

Origin	Transfer, *n* (%)	
	Grade 5 blastocysts	Grade 6 blastocysts
Vitrified grade 3 blastocysts	269 (51.34)	329 (25.97)
Vitrified grade 4 blastocysts	255 (48.66)	938 (74.03)
Total	524	1267

39.73% of grade 3 vitrified blastocyst grew into stage 5 blastocyst, and 48.60% grade 3 vitrified blastocyst cultured into stage 6 blastocyst. Amazingly, a similar trend occurred in grade 4 vitrified blastocyst. as presented in Table 5. We found that grade 4 vitrified blastocysts preferred to develop to stage 6 blastocysts by comparing the developmental outcome of stages 3 and 4 vitrified blastocysts (73.68% vs. 48.60%, $P < 0.001$). Conversely, we calculated the constituent ratio of the original blastocyst stage that developed into stage 5/6 transferred blastocyst. The stage 5 transferred blastocyst developed from grade 3 and 4 vitrified blastocysts, nearly in equal proportions (51.34% vs. 48.66%), while stage 6 transferred blastocyst mostly came from grade 4 vitrified blastocyst (25.97% vs. 74.03%), as listed in Table 6. Subsequently, we researched the transferred embryo quality and clinical outcomes and presented the results in Tables 7 and 8 and Fig. 3. The stage 6 transferred blastocyst that developed from stages 3 and 4 vitrified blastocysts were both significantly higher in clinical pregnancy rate compared with stage 5 transferred blastocyst that developed from grade 3 vitrified blastocyst (47.58% vs. 59.27%, $P = 0.004$; 47.58% vs. 62.79%, $P < 0.001$). Interestingly, a similar trend was found in the live birth rate. Additionally, stage 6 transferred blastocysts were significantly higher in clinical pregnancy rate than stage 5 transferred that both developed from grade 4 vitrified blastocysts (55.69% vs. 62.79%, $P = 0.039$). The stage 5 transferred blastocyst that developed from grade 4 vitrified blastocyst was markedly higher than that developed from grade 3 in live birth rate (45.88% vs. 35.32%, $P = 0.014$). At last, we explored the relationship between transferred embryo quality that developed from grade 3 + 4 vitrified blastocysts and clinical outcomes. The stage 6 transferred blastocyst was markedly higher in both clinical pregnancy and live birth rates compared to stage 5 transferred blastocysts (61.88% vs. 51.53%, $P < 0.001$; 50.91% vs. 40.46%, $P < 0.001$).

In this study, most of the vitrified grade 3 and grade 4 blastocyst developed into grade 6 blastocyst. Thus, the grade 6 blastocyst was a representative transferred blastocyst to be discussed.

By comparing Fig. 4a1 and a2, we found that grade 4 fresh blastocysts had a much more number and larger cell size of TE cells, which was in accordance with the opinion that the cell number was smaller in slower developing blastocyst than the faster developing blastocyst [18, 19]. By

Table 7 The clinical outcomes of transferred blastocysts that developed from different stages of vitrified embryo

Transf er, *n* (%)	Clinical pregnancy			Live birth		
	Vitrified grade 3 blastocysts	Vitrified grade 4 blastocysts	Overall (vitrified grade 3 + 4)	Vitrified grade 3 blastocysts	Vitrified grade 4 blastocysts	Overall (vitrified grade 3 + 4)
Grade 3 blastocyst	5 (12.20)	–	5 (12.20)	4 (9.76)	–	4 (9.76)
Grade 4 blastocyst	7 (18.42)	41 (51.25)	48 (40.68)	7 (18.42)	38 (47. 50)	45 (38.14)
Grade 5 blastocyst	128 (47.58)	142 (55.69)	270 (51.53)	95 (35.32)	117 (45.88)	212 (40.46)
Grade 6 blastocyst	195 (59.27)	589 (62.79)	784 (61.88)	162 (49.24)	483 (51.49)	645 (50.91)

Table 8 The comparison of clinical outcomes between different groups

Vitrified grade → Transfer grade	Clinical pregnancy		Live birth	
	P	OR (95%CI)	*P*	OR (95%CI)
3 → 5 vs 3 → 6	***P* = 0.004**	**0.62 (0.44 0.87)**	***P* = 0.001**	**0.56 (0.39 0.79)**
4 → 5 vs 4 → 6	***P* = 0.039**	**0.744 (0.56 0.99)**	*P* = 0.112	0.80 (0.60 1.06)
3 → 5 vs 4 → 5	*P* = 0.064	0.72 (0.50 1.03)	***P* = 0.014**	**0.64 (0.44 0.93)**
3 → 6 vs 4 → 6	*P* = 0.258	0.86 (0.66 1.12)	*P* = 0.482	1.09 (0.84 1.42)
3 → 5 vs 4 → 6	***P* < 0.001**	**0.54 (0.41 0.71)**	***P* < 0.001**	**0.51 (0.38 0.69)**
3 + 4 → 5 vs 3 + 4 → 6	***P* < 0.001**	**0.65 (0.53 0.81)**	***P* < 0.001**	**0.66 (0.53 0.81)**

Bold values indicate statistical significance

comparing b1 and b2, we observed that after AH, the cell proliferation of grade 3 vitrified-warmed blastocyst was decreased, and it hatched out at earlier time. By comparing c1 and c2, we found that during 16 h culturing, the grade 3 vitrified-warmed blastocyst had fewer cells and less contraction. By comparing d1 and d2, we observed that after 16 h culture, the grade 3 vitrified-warmed blastocyst had less chance to develop into a grade 6 blastocyst. Although the grade 6 blastocyst was developed, the cells was fewer and the volume was smaller.

Discussion

The retrospective study revealed that grade 4 blastocysts were more frequent along with high-grade ICM and TE cells. Secondly, the grade 4 vitrified–warmed blastocyst had significant potential to develop into a stage 6 blastocyst after further culturing for 16 h. Furthermore, the stage 6 transferred blastocyst was notably more excellent than the stage 5 transferred blastocyst in treatment outcomes. All above, the grade 4 vitrified blastocyst should be prioritized when warming a single blastocyst on day 5.

The present study suggested that the good-quality blastocyst was superior to the poor-quality blastocyst in both grade 3 and 4 vitrified–warmed groups. In other words, blastocyst with high-grade ICM and TE was more susceptible to high IVF success rate, which was consistent with our previously published literature [20]. It was confirmed that the optima ICM size, which was relatively large or slightly oval ICM, achieved the highest implantation rate [21]. Additionally, it was also revealed that the higher ICM grade, the better IVF treatment outcomes, including lower miscarriage rate and the higher clinical pregnancy and live birth rates [13, 22]. Furthermore, the high TE grade was vital in preventing pregnancy loss, obtaining a successful pregnancy, achieving a high live birth rate and sex of the offspring [12, 23–25]. In conclusion, ICM and TE grades were crucial in predicting IVF success rates.

Another interesting but also important finding was that the expansion and hatching stage should be considered first when transferring although the three parameters were important in predicting clinical outcomes [11]. The grade 3 blastocyst had significantly lower clinical pregnancy and live birth rates than the grade 4/5 blastocyst [13]. As expected, our present results were in accordance with the conclusion. Our data indicated that not only grade 4 was

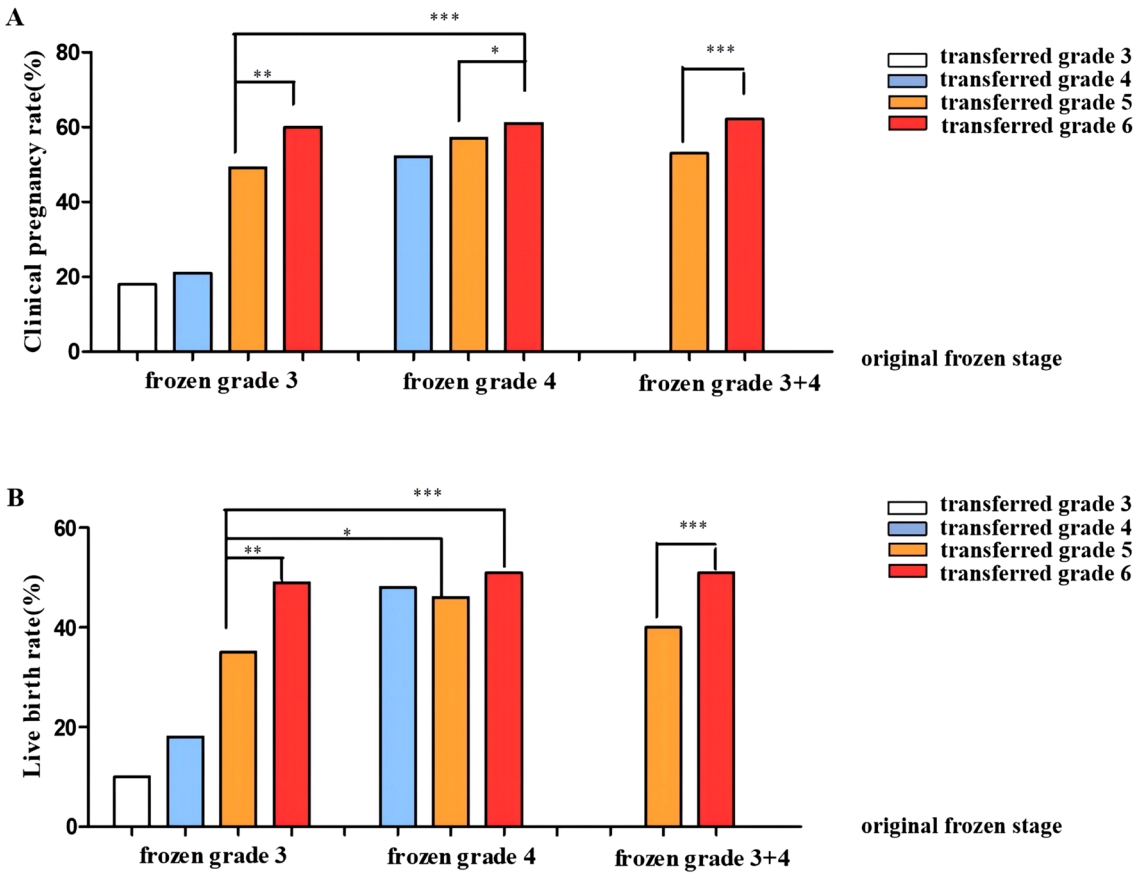

Fig. 3 Comparison of clinical outcomes after single blastocyst transfer according to embryo development stage. **A** Showed the comparison of clinical pregnancy rate: the grade 6 transferred blastocyst that developed from grades 3 and 4 vitrified blastocysts were both significantly higher compared with grade 5 transferred blastocyst that developed from grade 3 vitrified blastocyst; grade 6 transferred blastocysts were significantly higher in clinical pregnancy rate than grade 5 transferred that both developed from grade 4 vitrified blastocysts; the grade 6 transferred blastocyst was markedly higher than grade 5 transferred blastocysts those developed from grade 3 + 4 vitrified blastocysts. **B** Showed a similar trend in the live birth rate

superior to grade 3 vitrified–warmed blastocyst, but also, stage 6 was better than stage 5 transferred blastocysts in pregnancy and live birth rates. Thus, successful hatching played an important role in improving the IVF success rate [26]. Interestingly, we found that the high stage of expansion was more frequent along with high-grade ICM and TE. It was reported that the extent of blastocoel expansion is related to TE grade. The number and cohesiveness of TE cells contributed to pumping ions into the cavity, as well as, accumulating water in cells [27]. Conversely, the tight junctions of TE cells prevented blastocoel fluid and sodium ions from leaking [27]. All in all, the degree of blastocoel expansion depends on the functional TE. Thus, it could be concluded that the three parameters were interdependent.

Several possible mechanisms were proposed to clarify why the grade 4 vitrified blastocyst should be prioritized when warming a single blastocyst on day 5. First, the TE suffered from ultra-high temperature damage during the laser pulse AS and AH [28]. The larger the contact area of the laser pulse with TE, the greater damage was caused. However, the larger TE size and fewer TE cell numbers were observed in grade 3 vitrified–warmed blastocysts, which was in agreement with a previous study[18, 19], thus the grade 3 blastocyst suffered more damage during AS and AH. Second, the larger ICM/TE cells with the higher surface/volume ratio are more sensitive to osmotic stress and injury, thereby resulting in more intracellular ice crystals formed in grade 3 vitrified–warmed blastocyst [29]. Besides, the grade 4 blastocyst with smaller ICM/TE cell size could permit cryoprotectants more quickly permeate in and out. Thus, the grade 4 blastocyst was more tolerant of vitrification and cryoprotectant toxicity [30]. Third, according to previous literature [18, 19, 31], the ICM/TE cell number is a key indicator of blastocyst viability and quality, we also found that grade 4 blastocyst had more cells than grade 3 blastocyst of similar quality, thus, grade 4 blastocysts might have much more developmental potential than grade 3 blastocysts. The

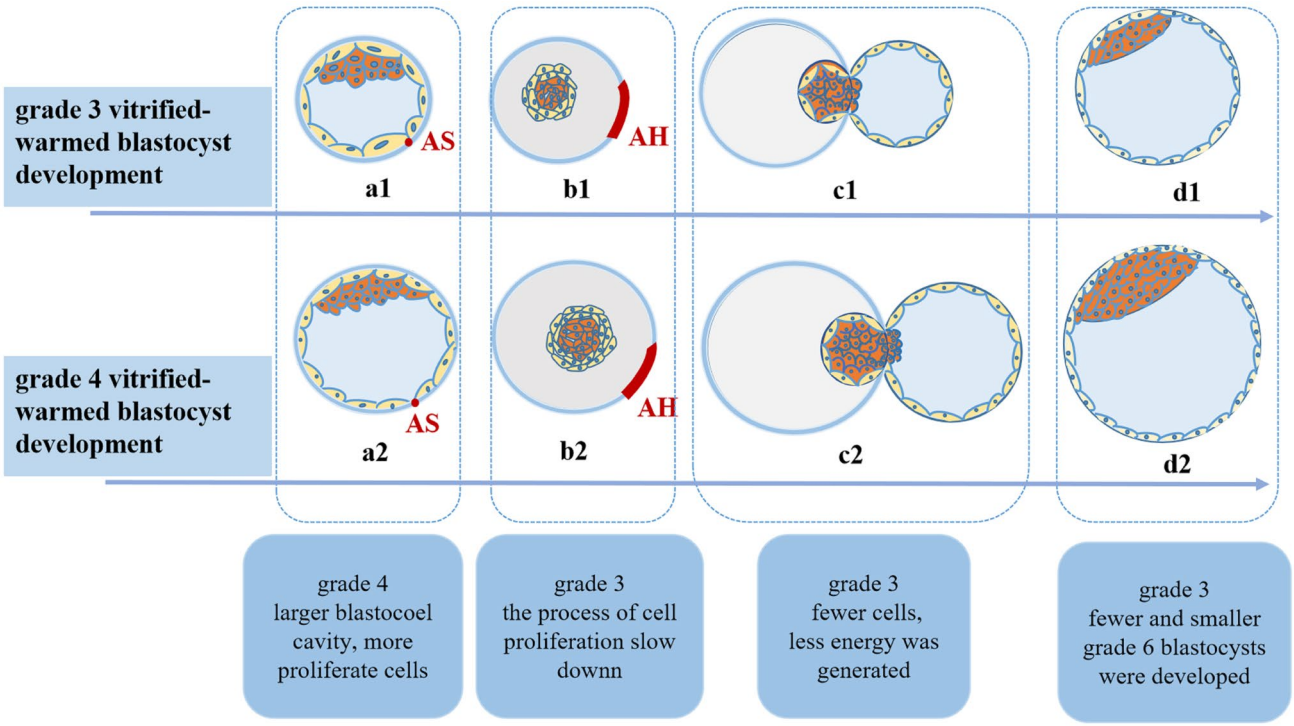

Fig. 4 Graphic representation of grades 3 and 4 blastocysts suffered from a series of development, including vitrified, warmed, and pre-transferred conditions. a1 and a2 represented grade 3 and 4 blastocyst that suffered from AS before vitrification, respectively. b1 and b2 represented the vitrified-warmed grade 3 and 4 blastocyst that suffered from AH, respectively. c1 and c2 represented the condition of vitrified-warmed grade 3 and 4 blastocyst during 16 h culturing, respectively. d1 and d2 represented the condition of vitrified-warmed grade 3 and 4 blastocyst after 16 h culturing, respectively

last but also the most important is the "embryonic escape hypothesis".

What is the embryonic escape hypothesis? We proposed it based on the following theoretical knowledge. In the normal development condition of grade 4 blastocysts, more and more fluid is accumulated in the blastocoel cavity [29], thus grade 4 blastocysts have a larger blastocoel cavity than grade 3 blastocysts (Fig. 4a1, a2). Furthermore, to prepare for releasing from zona pellucida and implanting into uterine endometrium successfully, the TE and ICM cells of grade 4 blastocysts had many more cell numbers to continue proliferating [32, 33] (Fig. 4a1, a2). When grade 3 blastocysts were vitrified and warmed, the AS and AH might lead to the breakage of zona pellucida at an earlier time, thus the process of cell proliferation became slowed down and blastocyst hatched out more easily (Fig. 4b1, b2). Therefore, fewer cells were generated and less energy was produced (Fig. 4c1, c2). Eventually, fewer and smaller grade 6 blastocysts were developed from grade 3 vitrified-warmed blastocysts after further culturing for 16 h (Fig. 4d1, d2), and the implantation rate was decreased. In short, the embryonic escape hypothesis was referred to as the grade 3 vitrified-warmed blastocyst skipped zona pellucida restriction at an earlier time, resulting in fewer TE and ICM cells, less energy and decreased implantation potential.

The main strength of this study was the combined analysis of the effect of vitrified and pre-transferred blastocyst quality on pregnancy outcomes. The second strength was that we first came up with the "embryonic escape hypothesis" to elucidate our findings. Third, the type of embryo culture media was identical and the assessment was evaluated by the same embryologist, thereby avoiding basis from different culture mediums and observers [34, 35]. However, the present study has a few limitations. Firstly, the single-center setting and small sample size weakened our evidence; thus, future multi-center analysis is needed. Secondly, the quantitative measurements were lacking to support the opinion that the cell size and cell number were larger in grade 4 blastocysts than in grade 3 blastocysts. Further scientific research is also urgent to be designed and analyzed to verify the "embryonic escape hypothesis". Finally, the optimized design could be to only include patients who had both grade 3 and grade 4 blastocysts transferred in separate SET, thus, comparisons would be paired using patients themselves as their own control.

Conclusions

This study recommends that grade 4 blastocysts should be prioritized to be thawed when facing single vitrified blastocysts on day 5 of transfer and the grade 3 blastocysts should be delayed for vitrification on day 5. Based on the results, grade 4 vitrified blastocysts were positively associated with high clinical pregnancy and live birth rates. However, no distinction was found in miscarriage rate, birth weight, or gestational age. Furthermore, our study demonstrated that grade 4 blastocysts on day 5 were more frequent, along with the good-quality ICM and TE, compared to grade 3 blastocysts on day 5. Conversely, grade 4 vitrified blastocysts on day 5 had a high potential to develop into stage 6 blastocysts after 16 h of culture. According to our observations, we first proposed the "embryonic escape hypothesis" to explain the mechanism. Moreover, providing advice to patients during FET treatment might be worthwhile for physicians.

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Author contributions JJ participated in the study design and drafted the article. XL performed the analysis and wrote the manuscript. QZ and LZ participated in the acquisition and analysis of data. HJ and XW reviewed the final article and made appropriate corrections and suggestions to improve it. SC and JZ are corresponding authors and they participated in the study design, did the final proof reading and confirmed the final version. All authors approved the final manuscript.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest There are no conflict of interest to declare.

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