Article

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Short-Term Post-Warming *In Vitro* Culture (<2 h vs. 2–4 h) Differentially Affects Vitrified Blastocyst Outcomes Depending on Transfer Number and Blastocyst Quality

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Background: Despite the wide application of vitrification-warming in assisted reproduction, the impact of post-warming *in-vitro* culture duration on the developmental potential of vitrified blastocysts remains controversial. Therefore, clarifying the optimal duration is crucial for better clinical outcomes. This study aimed to evaluate the effect of different short-term post-warming culture durations (<2 h vs. 2–4 h) on clinical outcomes and to explore optimal strategies based on transfer number and blastocyst quality.

Methods: This retrospective analysis included 1600 vitrified blastocyst warming-transfer cycles performed between 2021 and 2023, which were grouped according to post-warming culture duration (860 cycles: <2 h; 740 cycles: 2–4 h). Further subgrouping was conducted according to transfer number and blastocyst quality. Baseline data, clinical outcomes (such as implantation, clinical pregnancy, live birth and early abortion rates), and neonatal outcomes were compared. Statistical analyses were performed using the chi-square test for categorical variables and the Mann-Whitney U test for continuous variables, with Bonferroni correction for multi-group comparisons. A p value < 0.05 was considered statistically significant.

Results: There was no difference in baseline data between groups ($p > 0.05$). In single blastocyst transfers, the 2–4 h culture group demonstrated higher implantation rate (75.45% vs. 47.04%, $p < 0.001$), clinical pregnancy rate (75.45% vs. 47.04%, $p < 0.001$) and live birth rate (75.12% vs. 46.30%, $p < 0.001$). In double blastocyst transfers, the <2 h culture group had a higher live birth rate (46.74% vs. 35.34%, $p < 0.05$). Overall, the 2–4 h group exhibited a higher live birth rate compared to the <2 h culture group (67.97% vs. 46.40%, $p < 0.001$), and the D5 high-quality group had the highest live birth rate compared to other quality-related groups (61.93%, $p < 0.001$). For neonatal outcomes, the 2–4 h group displayed a higher singleton delivery rate (91.44% vs. 86.01%) and a lower multiple delivery rate compared to the <2 h culture group ($p = 0.008$), while other indicators showed no differences ($p > 0.05$).

Conclusion: The optimal post-warming culture duration may vary according to transfer number and blastocyst quality. Specifically, 2–4 h of culture appears beneficial for single blastocyst transfer, while <2 h may be preferable for double transfers. D5 high-quality blastocysts demonstrate optimal developmental potential. Culture duration does not compromise core neonatal health outcomes. These observations provide a basis for individualized transfer protocols to optimize clinical outcomes.

Keywords: vitrified blastocysts; post-warming culture duration; assisted reproduction; developmental potential; clinical outcomes; live birth rate

Introduction

Driven by continuous innovation, assisted reproductive technology (ART) has brought new hope for patients with infertility, with embryo cryopreservation technology serving as a critical pillar of this progress [1–3]. This technology not only effectively prevents and controls ovarian hyperstimulation syndrome (OHSS) in fresh cycle transfers but also optimizes the synchronization between the endometrium and embryos through elective transfer, ultimately leading to a significant improvement in the cumulative pregnancy rate [4,5]. Among various cryopreservation technologies, vitrification has gradually replaced conventional slow freezing as the preferred clinical method for embryo preservation due to its high recovery rate and minimal embryo damage, especially for blastocyst cryopreservation [6,7].

As a key step in embryo selection, blastocyst culture can further enrich embryos with superior developmental potential, reduce the risk of miscarriage, and improve the live birth rate [8]. Therefore, vitrified blastocyst transfer has been increasingly adopted in clinical practice [8,9]. How-

ever, the optimal duration of *in vitro* culture after blastocyst resuscitation remains controversial, and a unified clinical standard has yet to be defined. A study suggested that short-term post-warming culture is sufficient for transplantation, with same-day transfer after thawing having no effect on pregnancy outcomes [10], another study recommended extending culture to more than 2 h to better evaluate embryo viability and improve clinical results [11]. Some centers have even adopted an overnight culture strategy [12].

Notably, the embryonic development microenvironment differs between double blastocyst transfer and single blastocyst transfer. These embryos demonstrate weaker adaptability to *in vitro* culture conditions, rendering them vulnerable to additional damage during prolonged culture [13]. Existing studies still have limitations in terms of sample size, grouping design, and outcome indicator evaluation, making it difficult to fully guide the selection of culture duration in different transplantation scenarios.

This study retrospectively analyzed data from a large sample of blastocyst resuscitation and transplantation cycles. Patients were grouped by the *in vitro* culture duration after resuscitation, with further subgroup analysis based on the number and quality of transplanted blastocysts, to explore the effects of different culture durations on clinical pregnancy and neonatal outcomes. We aimed to clarify the optimal culture-duration protocol for different transplantation scenarios and to provide a scientific basis for the optimization of individualized clinical transplantation strategies.

Methods

Study Population

A retrospective analysis was conducted using clinical data from 1600 vitrified blastocyst warming-transfer cycles performed at the First People's Hospital of Foshan (Foshan Hospital Affiliated to Southern University of Science and Technology) from January 2021 to December 2023. This study was approved by the Ethics Review Committee of our hospital, and all patients signed the informed consents related to embryo cryopreservation, thawing, resuscitation and transplantation.

Inclusion criteria: (1) 20 $\leq$ Age $\leq$50 years; (2) Blastocysts cryopreserved on Day 5 (D5) or D6, meeting Grade 3 or above as per Gardner blastocyst grading criteria [14]; (3) No uterine abnormalities; (4) No transplantation cancellation after blastocyst thawing, with complete clinical data and follow-up until delivery.

Exclusion criteria: (1) Transplantation of cleavage-stage embryos after thawing; (2) Transplantation cancellation due to complications during the cycle; (3) Mental illness or inability to cooperate with follow-up.

Grouping Methods

Patients were divided into two groups based on the *in vitro* culture duration after blastocyst resuscitation: the $<$2 h group (culture $<$2 h, n = 860) and the 2–4 h group (culture for 2–4 h, n = 740). They were further subdivided according to the number of transplanted blastocysts into the single blastocyst group (1 blastocyst transplanted, n = 1283) and the double blastocyst group (2 blastocysts transplanted, n = 317). They were also classified by blastocyst quality into the D5 high-quality blastocyst group (n = 796), D5 non-high-quality blastocyst group (n = 114), D6 high-quality blastocyst group (n = 596), and D6 non-high-quality blastocyst group (n = 94). High-quality blastocysts were determined with reference to the Gardner blastocyst grading system [15]: the blastocyst stage was classified into Grade 3–6 (Grade 3: blastocoel filling the entire embryo; Grade 4: expanded blastocoel with a diameter larger than the original embryo and a thinned zona pellucida; Grade 5: trophoblast hatching but not detaching from the zona pellucida; Grade 6: blastocyst fully hatching and detaching from the zona pellucida). The inner cell mass (ICM) and trophoblast cells were graded A–C (Grade A: compact ICM with numerous cells, and abundant ectodermal cells forming a continuous epithelium; Grade B: relatively scattered ICM with few cells, and sparse ectodermal cells forming a loose epithelium; Grade C: ICM with scant cells, and ectoderm composed of a few large cells) [14]. Blastocysts of Grade 4BB and above were defined as high-quality, while other usable blastocysts (Grade 3BC and above) were considered non-high-quality.

Diagnostic and Therapeutic Methods

Ovarian Stimulation Protocols and Oocyte Retrieval

Individualized ovarian stimulation protocols were formulated based on patients' age and ovarian reserve function, including antral follicle count and follicle-stimulating hormone (FSH) level. Transvaginal B-mode ultrasound (Hitachi Aloka Medical, Ltd., Tokyo, Japan) was used for dynamic monitoring of follicular development. When at least 1–2 follicles reached a diameter $\geq$18 mm and the serum estrogen level aligned with follicular growth, 6000–10,000 U of human chorionic gonadotropin (HCG, Livzon, Guangdong, China) or 250µg of rHCG (Aizer, Merck Serono, Rome, Italy) was injected at 21:00 for ovulation triggering. Oocyte retrieval was performed 36–38 h after HCG injection by transvaginal B-ultrasound-guided follicle aspiration using an oocyte retrieval needle under intravenous or local anesthesia. Follicular fluid was aspirated to collect oocytes, which were temporarily stored in an incubator (37 °C, 5% CO_2; K-MINC-1000, COOK, Co., Sydney, Australia).

Fertilization and Embryo Culture

After washing, collected oocytes were subjected to conventional *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) based on sperm quality. For IVF, processed sperm and oocytes were co-cultured at a concentration of 1×10^5–2×10^5 sperm/oocyte. For ICSI, a single sperm with normal morphology and good vitality was selected and injected into the ooplasm. Pronucleus formation was observed 18–20 h post-fertilization to confirm normal fertilization (2 pronuclei, 2PN). Cleavage was monitored 24–26 h post-fertilization, and cleavage-stage embryo quality was evaluated at 68–70 h. High-quality cleavage-stage embryos (6–8 cells, fragmentation rate $<$20%) were selected for subsequent culture. Embryos meeting blastocyst culture criteria were transferred to G2Plus medium (10132 Lot# 26082, Vitrolife AB, Gothenburg, Sweden) and cultured in an incubator (K-MINC-1000, COOK, Co., Sydney, Australia; 5% CO_2, 95% air, 37 °C, saturated humidity) until D5 or D6. After blastocyst quality assessment, vitrification cryopreservation was performed.

Blastocyst Vitrification

A vitrification kit (VT621, Kitazato, Shizuoka, Japan) was used, and all cryopreservation reagents were pre-equilibrated at room temperature for 10–15 min. Blastocysts were first incubated in equilibration solution (ES solution) for 10–13 min, and then rapidly rinsed in vitrification solution (VS solution) within 60 s to avoid prolonged exposure to high-concentration cryoprotectants. Processed blastocysts were loaded onto cryocarriers (Cryotop-G, Kitazato, Shizuoka, Japan) and immediately immersed in liquid nitrogen at –196 °C for preservation. Cryopreservation information was recorded and properly archived.

Blastocyst Thawing and Resuscitation

With a vitrification kit (VT622, Kitazato, Japan), thawing reagents were prepared one day before transplantation. Thawing solution (TS solution) was pre-equilibrated in an incubator (37 °C, 5% CO_2) for over 30 min, while washing solution 1 (DS solution), washing solution 2 (WS1 solution), and washing solution 3 (WS2 solution) were equilibrated at room temperature for over 30 min. Cryocarriers were retrieved from the liquid nitrogen tank and rapidly immersed in 37 °C TS solution for 1 min to detach and resuscitate blastocysts. Subsequently, blastocysts were sequentially cultured in DS solution for 3 min, WS1 solution for 5 min, and WS2 solution on a 37 °C hot stage for 5 min to remove residual cryoprotectants. After resuscitation, assisted hatching (AH) was performed using a laser hatching instrument (LYKOS-001, Hamilton Thorne, Beverley, MA, USA), with a 10–15 µm diameter hole made at the equatorial region of the zona pellucida (ZP) to facilitate blastocyst expansion and implantation. Finally, blastocysts were transferred to pre-equilibrated G2Plus medium and cultured in an incubator (37 °C, 5% CO_2) for $<$2 h or 2–4 h before transplantation. Criteria for blastocyst survival were as follows: within 2–4 h post-thawing; the blastocoel re-expanded to a normal state; no obvious abnormalities in the morphology of the ICM and trophoblast cells.

Endometrial Preparation and Transplantation

Endometrial preparation protocols (natural cycle, hormone replacement cycle, or ovarian stimulation cycle) were selected based on patients' menstrual cycles. The natural cycle was applied to patients with regular menstruation (28–30 days) and normal ovulation: follicular development and endometrial thickness were monitored by B-ultrasound, and transplantation was performed 3–5 days after ovulation prediction when the follicle diameter was $\geq$18 mm. The hormone replacement cycle was utilized for patients with irregular menstruation or anovulation: 2–6 mg/d estradiol valerate (S300680, Progynova, Bayer Healthcare, Leverkusen, North Rhine-Westphalia, Germany) was administered orally on days 3–5 of menstruation, and progesterone (3736D3, dydrogesterone tablets, Duphaston, Solvay Pharmaceuticals B.V., Weesp, North Holland, Netherlands) was administered for endometrial transformation upon reaching endometrial thickness of $\geq$8 mm, following which transplantation was performed on days 5–7. During transplantation, a Cook Medical (K-J-SP-681700, Cook Medical, Bloomington, IN, USA) catheter was used to slowly deliver blastocysts to the mid-uterine cavity (1–2 cm from the fundus) under abdominal B-ultrasound guidance. The catheter was then withdrawn gently to avoid blastocyst loss, and patients could be discharged after 20–30 minutes of bed rest if no discomfort occurred.

Luteal Support and Pregnancy Assessment

Routine luteal support was administered after transplantation. Peripheral blood was collected on the 12th day post-transplantation, and serum β-human chorionic gonadotropin (β-HCG) level (00643953, SIEMENS Healthineers, Germany) was measured by chemiluminescence (SMART6500, Cosmai Biotechnology Co., Ltd., Chongqing, China). A β-HCG level $\geq$10 IU/L was defined as biochemical pregnancy [16]. Patients with positive biochemical pregnancy continued to receive luteal support, and transvaginal B-ultrasound was performed 4–5 weeks post-transplantation to observe the presence of a gestational sac, fetal bud, and primitive heart tube pulsation in the uterine cavity. Clinical pregnancy was confirmed when both a gestational sac and visible fetal heart pulsation were detected. Patients with clinical pregnancy underwent regular prenatal examinations and were followed up until delivery, with pregnancy outcomes and neonatal conditions recorded.

Outcome Measures

Baseline data were derived from the 1600 blastocyst resuscitation and transfer cycles included in this study, including age, infertility duration, body mass index (BMI), in-

Table 1. Comparison of baseline data between the <2 h group and the 2–4 h group.

Index	<2 h group (n = 860)	2–4 h group (n = 740)	Z/χ^2	p
Age (years)	34.00 (30.00, 37.00)	33.00 (30.00, 36.00)	–1.447	0.148
Infertility duration (years)	2.00 (1.00, 4.00)	3.00 (1.00, 4.00)	–0.377	0.706
BMI (kg/m^2)	21.62 (19.55, 24.03)	21.36 (19.53, 23.83)	–0.653	0.514
Infertility cause [N (%)]			1.792	0.181
Primary	400 (46.51)	369 (49.86)		
Secondary	460 (53.49)	371 (50.14)		
Endometrial thickness (mm)	10.90 (8.70, 13.00)	11.00 (9.20, 13.00)	–1.448	0.148
FSH level (U/L)	6.19 (5.09, 7.49)	6.05 (5.13, 7.23)	–1.492	0.136
Blastocyst development day [N (%)]			<0.001	0.990
D5	489 (56.86)	421 (56.89)		
D6	371 (43.14)	319 (43.11)		
Blastocyst quality [N (%)]			0.014	0.905
Non-high-quality blastocyst	111 (12.91)	97 (13.11)		
High-quality blastocyst	749 (87.09)	643 (86.89)		
Type of transplantation [N (%)]			2.932	0.087
Single blastocyst	676 (78.60)	607 (82.03)		
Double blastocyst	184 (21.40)	133 (17.97)		

Abbreviation: BMI, body mass index; FSH, follicle-stimulating hormone; D, Days. Quantitative data that did not conform to normal distribution were presented as median and interquartile range [M (P_{25}, P_{75})].

Table 2. Comparison of clinical outcomes in the single blastocyst group at different culture times [n (%)].

Index	<2 h group (n = 676)	2–4 h group (n = 607)	χ^2	p
Implantation rate	318 (47.04)	458 (75.45)	108.015	<0.001
Clinical pregnancy rate	318 (47.04)	458 (75.45)	108.015	<0.001
Live birth rate	313 (46.30)	456 (75.12)	110.641	<0.001
Early abortion rate	5 (1.57)	2 (0.44)	1.586	0.208

fertility cause (primary/secondary), endometrial thickness, FSH level, blastocyst development day, blastocyst quality, and number of transplanted blastocysts.

Clinical outcome indicators: Implantation rate = (number of gestational sacs in clinical pregnancy cycles / total number of transplanted embryos) × 100% [17]; live birth rate = (number of cycles with live births / total number of transplantation cycles) × 100% [18]; early abortion rate = (number of cycles with spontaneous abortion within 12 weeks of pregnancy / number of clinical pregnancy cycles) × 100% [17]; clinical pregnancy rate = (number of clinical pregnancy cycles / total number of transplantation cycles) × 100%.

Neonatal outcome indicators: Delivery mode (cesarean section/vaginal delivery), gestational age, birth weight, preterm birth rate (gestational age <37 weeks), birth weight classification (normal birth weight: 2500–4000 g; low birth weight: 1500–2499 g; very low birth weight: <1500 g; macrosomia: >4000 g), and gender ratio [19]. The neonatal outcome indicator data were obtained from the neonatal follow-up sample of this study.

Statistical Analysis

Statistical analyses were performed using SPSS 26.0 software (IBM-SPSS INC, Chicago, IL, USA). The Shapiro-Wilk test was employed to assess the normality of data distribution. Quantitative data that did not conform to normal distribution were presented as median and interquartile range [M (P_{25}, P_{75})], and comparisons between two groups were conducted via the Mann-Whitney U test. Qualitative data were expressed as rates or constituent ratios, with group comparisons analyzed using the chi-square (χ^2) test, and multiple intergroup comparisons were adjusted using the Bonferroni method. Categorical variables (birth weight classification) were amanyzed using Fisher's exact test. A p value < 0.05 was considered statistically significant.

Results

Comparison of Baseline Data Between the <2 h Group and the 2–4 h Group

No statistically significant differences were observed between the two groups in terms of age, infertility duration, BMI, infertility cause (primary/secondary), endometrial thickness and FSH level, ($p > 0.05$, Table 1); there was no significant differences observed in the number of transplantation cycles between the two groups in terms of blastocyst development day, blastocyst quality and the type of transplantion ($p > 0.05$, Table 1).

Table 3. Comparison of clinical outcomes in the double blastocyst group at different culture times [n (%)].

Index	<2 h group (n = 184)	2–4 h group (n = 133)	χ^2	p
Implantation rate	102 (27.72)	60 (22.56)	2.162	0.141
Clinical pregnancy rate	92 (50.00)	55 (41.35)	2.321	0.128
Live birth rate	86 (46.74)	47 (35.34)	4.120	0.042
Early abortion rate	6 (6.52)	8 (14.55)	2.572	0.109

Table 4. Comparison of live birth rates in different blastocyst quality groups [n (%)].

Index	Number of transplantation cycles	Live birth rate	χ^2	p
Culture time			75.300	<0.001
<2 h group	860	399 (46.40)		
2–4 h group	740	503 (67.97)		
Blastocyst quality			20.912	<0.001
D5 high-quality	796	493 (61.93)		
D6 high-quality	596	309 (51.85)*		
D5 non-high-quality	114	56 (49.12)		
D6 non-high-quality	94	44 (46.81)*		

Abbreviation: D, Days. Compared with D5 high-quality, *$p < 0.05$.

Comparison of Clinical Outcomes by Number of Transplanted Blastocysts

In the single blastocyst group, culture for 2–4 h resulted in higher implantation rate (75.45%), clinical pregnancy rate (75.45%) and live birth rate (75.12%) compared to culture for <2 h (47.04%, 47.04% and 46.30%, respectively) (Table 2, $p < 0.001$). The two groups did not differ significantly in the early abortion rate (1.57% vs. 0.44%, $p = 0.208$) (Table 2).

In the double blastocyst group, the live birth rate in the <2 h group (46.74%) was significantly higher than that in the 2–4 h group (35.34%) (Table 3, $p = 0.042$). There was no statistically significant difference in the implantation rate (27.72% vs. 22.56%, $p = 0.141$), clinical pregnancy rate (50.00% vs. 41.35%, $p = 0.128$) or the early abortion rate (6.52% vs. 14.55%, $p = 0.109$) between the two groups (Table 3).

Comparison of Live Birth Rates Among Groups With Different Blastocyst Quality

Relative to the <2 h group (46.40%), the 2–4 h group (67.97%) displayed a higher live birth rate (Table 4, $p < 0.001$). For blastocyst quality, the D5 high-quality blastocyst group exhibited the highest live birth rate (61.93%), followed by the D6 high-quality blastocyst group (51.85%), D5 non-high-quality blastocyst group (49.12%), and D6 non-high-quality blastocyst group (46.81%) (Table 4, $p < 0.001$).

Comparison of Neonatal Outcomes Between Groups With Different Culture Durations

No statistically significant differences were observed between the <2 h group (n = 429) and 2–4 h group (n = 526) in terms of delivery mode, gender ratio, gestational age, and distribution of birth weight classification (Table 5, $p > 0.05$). The singleton delivery rate (91.44%) was higher, yet the multiple delivery rate (8.56%) was lower in the 2–4 h group compared with the <2 h group (86.01%; 13.99%) (Table 5, $p = 0.008$).

Discussion

Vitrified blastocyst transfer, a core assisted reproductive protocol [20], enriches high-potential embryos via morphological selection, and thus improves pregnancy outcomes [21]. The duration of post-warming *in vitro* culture plays a vital role in transplantation, directly affecting embryonic repair and developmental potential; however, a unified standard has yet to be established [10]. Based on 1600-cycle large-sample data, with stratification by blastocyst number and quality, this study identified significant differential effects of culture duration on clinical outcomes, providing evidence for individualized protocol optimization.

In single blastocyst transfer, the implantation rate, clinical pregnancy and live birth rate in the 2–4 h culture group were significantly higher than those in the <2 h group. Consistent with our findings, Tao *et al.* [11] reported that post-thaw culture of 2–5 h improves clinical pregnancy and implantation rates. Short-term culture (<2 h) may lead to embryos entering the uterine cavity before complete damage repair and full recovery of developmental activity, resulting in outcome differences [22]. Intriguingly, double blastocyst transfer showed the opposite result: the live birth rate was significantly higher in the <2 h group than in the 2–4 h group, suggesting that embryos in double blastocyst transfer were more sensitive to transplantation timing. Notably, the *in vitro* environment cannot replicate the physiological conditions of the uterine cavity, and pro-

Table 5. Comparison of neonatal outcomes in different culture time groups.

Index	<2 h group (n = 429)	2–4 h group (n = 526)	Z/χ^2	p
Delivery results [n (%)]			7.122	0.008
Singleton delivery	369 (86.01)	481 (91.44)		
Multiple delivery	60 (13.99)	45 (8.56)		
Gender [n (%)]			1.104	0.293
Male	226 (52.68)	295(56.08)		
Female	203 (47.32)	231 (43.92)		
Delivery mode [n (%)]			0.049	0.825
Cesarean section	285 (66.43)	353 (67.11)		
Vaginal delivery	144 (33.57)	173 (32.89)		
Gestational age (weeks)	38.00 (37.00, 39.00)	38.00 (37.00, 39.00)	–0.438	0.661
Birth weight (g)	3130.00 (2800.00, 3400.00)	3180.00 (2860.00, 3525.00)	–1.924	0.054
Preterm birth [n (%)]	56 (13.05)	56 (10.65)	1.323	0.250
Birth weight classification [n (%)]			5.478	0.130
Normal birth weight	352 (82.05)	457 (86.88)		
Very low birth weight	1 (0.23)	3 (0.57)		
Low birth weight	58 (13.52)	50 (9.51)		
Macrosomia	18 (4.20)	16 (3.04)		

longed exposure may trigger oxidative stress and metabolic disorders to affect implantation. Overall, the total live birth rate in the 2–4 h group remained higher than that in the <2 h group.

In terms of blastocyst quality stratification, the D5 high-quality blastocyst group had the highest live birth rate, followed by the D6 high-quality group, the D5 non-high-quality group, and the D6 non-high-quality group. This finding aligned with the core value of the Gardner blastocyst grading system: D5 high-quality blastocysts possess a mature, tightly organized ICM and trophoblast cells, with greater stress resistance and repair capacity [23,24]. Andrabi *et al*. [14] explored whether developmental potential affects clinical outcomes when transferring good-quality D5 and D6 blastocysts and found that D5 blastocysts are associated with significantly higher live birth rates.

We analyzed the impact of post-warming culture duration on neonatal outcomes, and demonstrated that the singleton delivery rate was higher, yet the multiple delivery rate was lower in the 2–4 h group compared with the <2 h group, with no differences in delivery mode, gestational age, and birth weight classification. An existing study confirmed that varying post-thaw culture durations (even longer) make no impact on neonatal outcomes, live birth and clinical pregnancy rates [25]. Consistently, we corroborated that culture duration mainly affected implantation rather than intrauterine development, thereby verifying the safety of our scenario-specific protocols. Additionally, the higher singleton rate in the 2–4 h group (attributable to its higher implantation rate in single blastocyst transfers) ensured pregnancy success while reducing the risks of multiple gestation, aligning with the "safe and efficient" goals of ART.

Notably, this study has limitations. As a single-center retrospective study, it was subject to inevitable selection bias. Therefore, the generalizability of the results requires verification by multi-center, large-sample prospective studies. Additionally, the lack of long-term neonatal health follow-up data precluded a comprehensive assessment of the impact of culture duration on long-term offspring outcomes, such as growth and intellectual development, which need further tracking and evaluation.

Conclusion

In summary, this study confirms that post-warming *in vitro* culture duration impacts clinical outcomes in a scenario-specific manner, depending on the number and quality of transplanted blastocysts. Specifically, 2–4 h culture is recommended for single blastocyst transfer to maximize the developmental potential of high-quality embryos, where <2 h culture is advised for double blastocyst transfer to minimize *in vitro* damage to low-quality embryos. D5 high-quality blastocysts should be considered the preferred choice. Future research should expand sample size, include special populations, conduct multi-center prospective trials, and establish long-term follow-up cohorts to provide comprehensive evidence for refined, individualized ART strategies and to better safeguard maternal-infant safety.

Availability of Data and Materials

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

Author Contributions

JZH: Conceptualization, Methodology, Validation, Writing - original draft, Writing - review & editing. JHH: Conceptualization, Investigation, Validation, Writing

- original draft, Writing - review & editing. BCM: Data curation, Formal analysis, Methodology, Writing - original draft, Writing - review & editing. CC: Investigation, Formal analysis, Supervision, Writing - original draft, Writing - review & editing. XLL: Data curation, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. ZWY: Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. WMM: Data curation, Project administration, Writing - original draft, Writing - review & editing. All authors gave final approval of the version to be published. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Review Committee of the First People's Hospital of Foshan (Ethics Review (Research) No. 97 (2026)), and all patients were fully informed and signed the informed consent. The study adhered to the Declaration of Helsinki.

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Conflict of Interest

The authors declare no conflict of interest.

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