INTRODUCTION

After the first report of a live birth achieved using transfer of frozen embryos\(^1\) and the success of vitrification,\(^2\) embryo cryopreservation is widely used at in vitro fertilization (IVF) centers. This has resulted in a dramatic increase in the number of pregnancies conceived after frozen embryo transfer (FET).\(^3\) FET avoids the detrimental effects of high-dose hormones used for controlled ovarian stimulation on the endometrium and prevents ovarian hyperstimulation syndrome (OHSS).\(^4\) A number of studies have suggested that fertility outcomes are comparable after FET compared with fresh embryo transfer.\(^5-7\)

As a result, embryo cryopreservation has become a fundamental part of assisted reproduction technology (ART).

After thawing, embryos can be cultured \textit{in vitro} until FET. The purpose of this culture is to help the embryo recover, and stabilize the osmotic pressure after freezing and thawing. Although the
embryo culture medium has been optimized to resemble that of the uterus, there is still the potential for this post-thawing culture to have adverse effects on the embryo.\textsuperscript{8} It was suggested that embryos might respond poorly to the culture medium and be susceptible to oxidative stress, leading to lower implantation rates.\textsuperscript{9} Some studies have shown that post-warming embryo culture for 2–5 h results in higher implantation and live birth rates than culturing overnight.\textsuperscript{10,11} However, other studies showed no differences in clinical outcomes between the short and long culture duration groups.\textsuperscript{12,13} Zhu and colleagues evaluated the effect of different post-warming culture durations on outcomes after FET of day-3 embryos and showed that embryo implantation and live birth rates tended to increase as the duration of post-warming culture increased from 2–8 h.\textsuperscript{14}

This study evaluated the influence of post-warming culture time on the live birth rate of FET cycles using day-3 or day-5 embryos.

2 | MATERIALS AND METHODS

2.1 | Study design

This multicenter, retrospective cohort study was performed at IVFMD, My Duc Hospital, and IVFMD Phu Nhuan, My Duc Phu Nhuan Hospital, both in Vietnam. The study was approved by the Medical Ethics Committee at My Duc Hospital, Ho Chi Minh City, Vietnam (05/21/DD-BVMD) on 20th April 2021.

2.2 | Study population

Data were extracted from the database of each center. All ART cycles undergoing intracytoplasmic sperm injection (ICSI) and FET where ≤2 day-3 or day-5 embryos were transferred between October 2019 and October 2020 were evaluated for eligibility. Cycles performed in women aged <39 years, with <4 previous IVF cycles, and <4 embryo transfers were eligible for the analysis. Cycles with in vitro maturation (IVM), oocyte donation or preimplantation genetic testing, and those in women with uterine abnormalities were excluded. Cycles with <50% of cells intact after warming were also not eligible. Post-warming culture time was defined as the time from warming to embryo transfer (in hours). Electronic embryo monitoring was used to record real-time data on warming and embryo transfer time. Eligible cycles were divided into four groups based on the quartile of time between warming and embryo transfer (post-warming culture time) for each stage of embryo.

2.3 | Ovarian stimulation

All patients were treated with a gonadotropin-releasing hormone (GnRH) antagonist protocol, as described previously.\textsuperscript{15} Recombinant follicle-stimulating hormone (FSH) was given on day 2 or day 3 of the menstrual cycle for 5 days. The starting dose was individualized for each patient based on the anti-Müllerian hormone level, with subsequent dosage titration based on the treating physician’s clinical judgment. Follicular development was monitored by ultrasound scanning and by the measurement of estradiol and progesterone levels, starting on day 5 of stimulation. Scanning and hormonal measurements were repeated every 2–3 days, depending on follicle size. A GnRH antagonist was routinely used on day 5 until the day of triggering. Criteria for human chorionic gonadotropin (hCG) triggering were the presence of at least three leading follicles with a diameter of 17 mm. In women with an excessive follicular response (≥15 follicles of ≥12 mm in diameter), triggering was performed with triptorelin 0.2 mg when there were at least two leading follicles of 17 mm in diameter. Oocyte retrieval was performed 36 h after triggering.\textsuperscript{15}

2.4 | Insemination and embryo culture

Insemination was performed by ICSI at 39–41 h after the triggering. Only metaphase II (MII) oocytes were used. A fertilization check was performed 16–18 h after insemination. Embryos were cultured in Global Total LP (LifeGlobal, Denmark) covered with paraffin oil (ORIGIO, Denmark) at 37°C in 5% carbon dioxide and 5% oxygen. After evaluation on day 3, embryos could be cultured to day 5 with a renewable media or all vitrified for transfer in subsequent FET cycles.

Embryo evaluation was performed at a fixed time point of 66 ± 1 h using the Istanbul consensus\textsuperscript{16} for day 3 and 116 ± 2 h using Gardner consensus\textsuperscript{17} for day 5 after ICSI. A top-quality embryo was defined as at least 6 blastomeres with <25% fragmentation; or had a grade A or B of inner cell mass and trophectoderm and at least grade 2 of blastocoel expansion for day-3 and day-5 evaluation, respectively.\textsuperscript{16,17} Embryos were vitrified with a maximum of two embryos per cryotec (Cryotech), based on the quality of embryos and couples’ preferences. Embryos were placed into equilibration solution following vitrification solution according to the Cryotech instructions.

2.5 | Warming protocol

The warming procedure was performed using a warming kit (Cryotech). Embryos on the cryotec were put into warming solution for 1 minute, followed by diluent solution for 3 minutes, and washing solution for 6 minutes. After warming, embryos were immediately evaluated for warm morphological survival. Embryos with at least 50% of their cells intact were considered to have survived and were eligible for transfer. Survival rate was calculated as the number of surviving embryos after warming per total number of embryos warmed.

2.6 | Frozen embryo transfer

In a FET cycle, the endometrium was prepared using oral estradiol valerate 8 mg/day starting from the second or third day of the
menstrual cycle. Endometrial thickness was monitored from day six onward, and vaginal progesterone was started when endometrial thickness reached ≥8 mm. A maximum of two embryos was thawed on the day of embryo transfer, 3 or 5 days after the start of progesterone. Luteal phase support was provided with exogenous estradiol and vaginal progesterone, continued until the seventh week of gestation. Serum hCG level was measured 2 weeks after embryo transfer, and, if positive, an ultrasound scan of the uterus was performed at 7 and 12 weeks' gestation.

2.7 | Outcomes

The primary outcome was the live birth rate after FET. Live birth was defined as the birth of at least one baby after 24 weeks’ gestation that showed any sign of life (twins as a single count). Secondary outcomes were rates of positive hCG, clinical pregnancy, ongoing pregnancy, implantation, miscarriage, and ectopic pregnancy.

2.8 | Statistical analysis

Baseline data were presented using descriptive statistics (mean and standard deviation for normally distributed variables, or median and interquartile range for skewed variables). Categorical data were presented as numbers (%). Patients were divided into four quartiles based on post-warming culture time (with cut-offs at the 25th, 50th, and 75th percentiles). Differences between groups were analyzed using one-way analysis of variance (ANOVA) with post hoc Tukey HSD test or Kruskal–Wallis test for normally distributed or skewed variables, respectively, and the Chi-square test for categorical variables. Univariable and multivariable logistic regression analyses were performed to identify factors associated with live birth. All variables with a p-value of <0.25 in the univariate analysis were included in the multivariable analysis. All analyses were performed using the R statistical programme (R version 4.1.0; ©2021 The R Foundation for Statistical Computing). Statistical significance was defined as p < 0.05.

3 | RESULTS

3.1 | Study population

From a total of 6759 FET cycles performed between October 2019 and October 2020, this analysis included 2009 women with 2049 ICSI cycles and 2548 FET cycles (Figure 1). Patients were lean and relatively young, the majority had primary infertility, and nearly 80% were undergoing their first IVF cycle (Table 1). The mean total FSH dosage used for controlled ovarian stimulation was 2325 IU. More
patients had day-5 than day-3 embryos cryopreserved (64.2% vs. 35.8%, respectively) (Table 2). Patient characteristics, including age, body mass index (BMI), anti-Müllerian hormone (AMH) level, and antral follicle count (AFC), were similar across the four post-warming culture time quartiles in both day-3 and day-5 transfer groups (Table 3).

### 3.2  Fertility outcomes

There were no significant differences between post-warming culture time quartile groups with respect to the number of embryos thawed, the number of embryos transferred, and the number of top-quality embryos transferred; results were similar for day-3 and day-5 embryos (Table 3). The survival rate was not significantly different between quartile groups for either day-3 or day-5 embryos. When day-3 embryos were transferred, the live birth rate did not differ significantly among the four post-warming culture time quartiles (26.8%, 31.2%, 32.5%, and 34.6% in quartiles 1, 2, 3, and 4, respectively) (Table 3). Live birth rates after transfer of day-5 embryos were above 50% for all post-warming culture time quartiles, with no significant between-quartile differences (Table 3). All other fertility outcomes were also comparable between the different post-warming culture time quartiles for both day-3 and day-5 embryos (Table 3).

### 3.3  Predictors of live birth

Independent predictors of live birth on multivariate analysis were patient age and the stage of embryo(s) transferred (i.e., day-3 or day-5 embryos). Post-warming culture time was not a significant predictor of live birth (Table 4).

### 4  DISCUSSION

Our study showed that the culture time from warming to embryo transfer was not associated with the live birth rate in FET cycles. Previous retrospective studies showed no significant difference in clinical pregnancy rate and live birth rate between short culture (2–4 h) and long culture (20–24 h) after embryo warming. However, these studies only evaluated these outcomes for cleavage stage (day-3) embryos, and there is a lack of corresponding data relating to blastocyst stage (day-5) embryo transfer. This was the rationale for including both cleavage stage and blastocyst stage embryos in our study. Blastocyst transfer is a strategy used at our center to adequately select embryos to reduce the multiple pregnancy rate without compromising pregnancy outcomes. In this study, blastocyst transfers comprised approximately two-thirds of all FET.
| Post-warming culture time quartiles | 0.07–1.39 h | 1.4–2.29 h | 2.3–3.29 h | 3.3–6.1 h | p-value |
|-----------------------------------|-------------|------------|------------|------------|---------|
| Day-3 embryo transferred          |             |            |            |            |         |
| (n = 209)                         | (n = 205)   | (n = 243)  | (n = 228)  |            |         |
| Age, years                        | 31.7 ± 3.80 | 32.0 ± 3.96| 31.2 ± 3.78| 32.2 ± 3.83| 0.055   |
| Body mass index, kg/m²            | 21.1 ± 2.71 | 21.3 ± 2.93| 21.2 ± 2.57| 21.1 ± 2.43| 0.897   |
| Anti-Müllerian hormone, ng/mL     | 2.50 [1.32; 3.94] | 2.53 [1.52; 4.09] | 2.38 [1.46; 4.33] | 2.41 [1.39; 3.70] | 0.463   |
| Antral follicle count, n          | 11.0 [7.00; 17.0] | 12.0 [8.00; 18.0] | 12.0 [7.00; 18.0] | 12.0 [8.00; 18.0] | 0.355   |
| PCOS, n (%)                       | 13 (6.2)    | 14 (6.8)   | 22 (9.0)   | 8 (3.5)    | 0.108   |
| Endometrial thickness, mm         | 10.6 ± 1.2  | 10.5 ± 1.0 | 10.5 ± 1.2 | 10.6 ± 1.3 | 0.466   |
| Number of embryos thawed         | 2.00 [2.00; 2.00] | 2.00 [1.00; 2.00] | 2.00 [1.00; 2.00] | 2.00 [2.00; 2.00] | 0.157   |
| Survival rate a                   | 363/368 (98.6) | 356/356 (100) | 408/412 (99.0) | 406/407 (99.8) | 0.759   |
| Number of embryos transferred     | 2.00 [2.00; 2.00] | 2.00 [1.00; 2.00] | 2.00 [1.00; 2.00] | 2.00 [2.00; 2.00] | 0.167   |
| Number of top-quality embryos transferred | 2.00 [1.00; 2.00] | 2.00 [1.00; 2.00] | 2.00 [1.00; 2.00] | 2.00 [1.00; 2.00] | 0.378   |
| Positive hCG, n (%)               | 81 (38.8)   | 87 (42.4)  | 106 (43.6) | 103 (45.2) | 0.575   |
| Clinical pregnancy, n (%)         | 62 (29.7)   | 75 (36.6)  | 88 (36.2)  | 91 (39.9)  | 0.159   |
| Implantation, n (%)               | 22.2 (39.2) | 26.6 (37.9) | 26.7 (39.7) | 28.9 (39.5) | 0.344   |
| Miscarriage <12 weeks, n (%)      | 6 (2.9)     | 7 (3.4)    | 6 (2.5)    | 9 (4.0)    | 0.816   |
| Ectopic pregnancy, n (%)          | 1 (0.5)     | 4 (2.0)    | 3 (1.2)    | 3 (1.3)    | 0.626   |
| Ongoing pregnancy, n (%)          | 55 (26.3)   | 64 (31.2)  | 79 (32.5)  | 79 (34.6)  | 0.287   |
| Miscarriage <24 weeks, n (%)      | 0 (0)       | 0 (0)      | 0 (0)      | 0 (0)      | -       |
| Live birth, n (%)                 | 56 (26.8)   | 64 (31.2)  | 79 (32.5)  | 79 (34.6)  | 0.345   |
| Day-5 embryo transferred          |             |            |            |            |         |
| (n = 428)                         | (n = 432)   | (n = 394)  | (n = 409)  |            |         |
| Age, years                        | 30.8 ± 3.75 | 30.7 ± 3.49| 30.4 ± 3.57| 30.8 ± 3.73| 0.336   |
| Body mass index, kg/m²            | 21.5 ± 2.83 | 21.3 ± 2.68| 21.7 ± 2.74| 21.2 ± 2.66| 0.083   |
| Anti-Müllerian hormone, ng/mL     | 4.08 [2.51; 6.48] | 4.01 [2.52; 6.56] | 4.38 [3.02; 6.83] | 3.96 [2.48; 5.73] | 0.042   |
| Antral follicle count, n          | 17.0 [11.0; 25.0] | 17.0 [11.0; 23.0] | 20.0 [14.0; 26.0] | 18.0 [11.0; 25.2] | 0.045   |
| PCOS, n (%)                       | 72 (16.8)   | 74 (17.1)  | 76 (19.3)  | 61 (14.9)  | 0.434   |
| Endometrial thickness, mm         | 10.6 ± 1.2  | 10.5 ± 1.2 | 10.4 ± 1.2 | 10.5 ± 1.2 | 0.056   |
| Number of embryos thawed         | 1.00 [1.00; 1.00] | 1.00 [1.00; 1.00] | 1.00 [1.00; 1.00] | 1.00 [1.00; 2.00] | 0.424   |
| Survival rate a                   | 519/522 (99.4) | 532/534 (99.6) | 487/489 (99.6) | 516/518 (99.6) | 0.983   |
| Number of embryos transferred     | 1.00 [1.00; 1.00] | 1.00 [1.00; 1.00] | 1.00 [1.00; 1.00] | 1.00 [1.00; 2.00] | 0.41    |
| Number of top-quality embryos transferred | 1.00 [1.00; 1.00] | 1.00 [1.00; 1.00] | 1.00 [1.00; 1.00] | 1.00 [1.00; 1.00] | 0.401   |
| Positive hCG, n (%)               | 306 (71.5)  | 292 (67.6) | 260 (66.0) | 291 (71.1) | 0.241   |
| Clinical pregnancy, n (%)         | 263 (61.4)  | 251 (58.1) | 229 (58.1) | 258 (63.1) | 0.362   |
| Implantation, n (%)               | 59.3 (49.5) | 55.1 (49.6) | 55.1 (49.2) | 59.3 (48.0) | 0.378   |
| Miscarriage <12 weeks, n (%)      | 28 (6.5)    | 25 (5.8)   | 22 (5.6)   | 23 (5.6)   | 0.929   |
| Ectopic pregnancy, n (%)          | 8 (1.9)     | 5 (1.2)    | 7 (1.8)    | 2 (0.5)    | 0.276   |
| Ongoing pregnancy, n (%)          | 227 (53.0)  | 221 (51.2) | 200 (50.8) | 233 (57.0) | 0.265   |
| Miscarriage <24 weeks, n (%)      | 2 (0.5)     | 4 (0.9)    | 1 (0.3)    | 1 (0.2)    | 0.551   |
| Live birth, n (%)                 | 226 (52.8)  | 217 (50.2) | 199 (50.5) | 232 (56.7) | 0.216   |

Note: Values are presented as mean ± standard deviation, median [25th; 75th percentile], or n (%). The p-values were calculated using one-way analysis of variance or Chi-square test.
Abbreviations: hCG, human chorionic gonadotropin; PCOS, polycystic ovary syndrome.

aTotal number of surviving embryos/total number of thawing embryos.
Table 4: Univariate and multivariate regression analysis of factors affecting live birth after frozen embryo transfer cycles

| Factor                         | No live birth (n = 1396) | Live birth (n = 1152) | Odds ratio (95% CI), p-value |
|--------------------------------|--------------------------|-----------------------|----------------------------|
|                                | Univariate               | Multivariate          |                            |
| Age, years                     | 31.4 ± 3.8               | 30.6 ± 3.6            | 0.94 (0.92–0.96), <0.001   |
|                                | 1.02 (1.01–1.03), <0.001  | 1.00 (0.99–1.01), 0.972 |
| Body mass index, kg/m²         | 21.3 ± 2.8               | 21.3 ± 2.6            | 1.00 (0.97–1.03), 0.845    |
|                                | Ref.                     | Ref.                 |                            |
| Anti- Müllerian hormone, ng/mL | 3.4 [1.9; 5.1]           | 3.8 [2.3; 5.6]        | 1.04 (1.01–1.07), 0.007    |
|                                | 0.99 (0.94–1.05), 0.843  | Ref.                 |                            |
| Antral follicle count, n       | 14.0 [9.0; 21.0]         | 16.0 [10.0; 24.0]     | 1.04 (1.01–1.03), <0.001   |
|                                | 1.02 (1.01–1.03), <0.001  | 1.00 (0.99–1.01), 0.972 |
| PCOS, n (%)                    |                          |                       |                            |
| No                             | 1219 (87.3)              | 989 (85.9)            | Ref.                       |
| Yes                            | 177 (12.7)               | 163 (14.1)            | 1.14 (0.90–1.43), 0.278    |
|                                | 0.84 (0.54–1.31), 0.446  | Ref.                 |                            |
| Number of embryos transferred  | 1.0 [1.0; 2.0]           | 1.0 [1.0; 2.0]        | 0.76 (0.64–0.89), 0.001    |
|                                | 1.12 (0.90–1.39), 0.33   | Ref.                 |                            |
| Number of good embryos transfer| 1.0 [1.0; 1.0]           | 1.0 [1.0; 1.0]        | 1.01 (0.88–1.15), 0.933    |
|                                | Ref.                     | Ref.                 |                            |
| Stage of embryos transferred, n (%) |                       |                       |                            |
| Day-3                          | 607 (43.5)               | 278 (24.1)            | 2.42 (2.04–2.87), <0.001   |
|                                | 2.59 (2.05–3.27), <0.001  | Ref.                 |                            |
| Day-5                          | 789 (56.5)               | 874 (75.9)            | Ref.                       |
|                                | Ref.                     | Ref.                 |                            |
| Post-warming culture time quartile, n (%) |                   |                       |                            |
| 0.07–1.39 h                    | 355 (25.4)               | 282 (24.5)            | Ref.                       |
|                                | 0.99 (0.80–1.24), 0.955  | Ref.                 |                            |
| 1.4–2.29 h                     | 356 (25.5)               | 281 (24.4)            | 0.97 (0.78–1.22), 0.822    |
|                                | 1.00 (0.76–1.31), 0.982  | Ref.                 |                            |
| 2.3–3.29 h                     | 359 (25.7)               | 278 (24.1)            | 1.20 (0.96–1.50), 0.104    |
|                                | 1.18 (0.90–1.55), 0.232  | Ref.                 |                            |
| 3.3–6.1 h                      | 326 (23.4)               | 311 (27.0)            | Ref.                       |
|                                | Ref.                     | Ref.                 |                            |

Note: Values are presented as mean ± standard deviation, median [25th; 75th percentile], or n (%). Odds ratio (95% CI) and p-values were calculated using univariable and multivariable logistic regression models. Abbreviations: CI, confidence interval; PCOS, polycystic ovary syndrome.
6 h did not affect the ongoing pregnancy and live birth rates after blastocyst transfers. Therefore, we agree that there is no need to prolong the post-warming culture time for blastocyst-stage embryos if at least one top-quality embryo is transferred. This helps to make the procedure more convenient for patients.

Unlike other studies,^{10–13,21,24} our study focused on short-term post-warming culture times on the day of embryo thawing (0.07–1.39, 1.4–2.29, 2.3–3.29 and 3.3–6.1 h in quartiles 1, 2, 3, and 4, respectively). We wanted to investigate whether differences between these short periods of culture time affected the live birth rate after FET because sticking to an exact time from embryo warming to embryo transfer might be a challenge for a busy center like ours.

Many retrospective studies have investigated the influence of post-warming culture time on clinical outcome,^{10–14} but the results are conflicting, and most focused on the effect of patient age and lacked comprehensive baseline characteristics from oocyte retrieval cycles. Despite the limitations inherent in the retrospective design of our study, we included comprehensive baseline and treatment cycle characteristics for all included patients. In addition, we used univariate and multivariate regression analysis to identify factors associated with successful live birth. Another limitation of our study is that the results are only applicable to patients with similar characteristics to our study population (i.e., young, lean women of Vietnamese ethnicity). Future trials should be prospectively designed to determine the effect of different culture time durations after embryo warming. This will provide robust data to support the findings of our retrospective analysis.

In conclusion, post-warming culture time did not affect the live birth rate in FET cycles. Therefore, IVF centers should consider scheduling workflows to maximize patient convenience and comfort.

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CONFLICT OF INTEREST

LNV has received speaker and conference fees from Merck; and grant, speaker, and conference fees from Merck Sharp & Dohme and Ferring. TMH has received speaker fees from Merck, Merck Sharp & Dohme, and Ferring. HHP, TMV, CHN, DPN, AHL, and TDP have no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years and no other relationships or activities that could appear to have influenced the submitted work.

HUMAN RIGHTS STATEMENT AND INFORMED CONSENT

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and its later amendments.

ANIMAL STUDIES

This study does not contain any studies with animal subjects performed by any of the authors.

APPROVAL BY THE ETHICS COMMITTEE

The study was approved by the Medical Ethics Committee at My Duc Hospital, Ho Chi Minh City, Vietnam (05/21/DD-BVMD) on 20th April 2021.

CLINICAL TRIAL REGISTRY

This was not a clinical trial.

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