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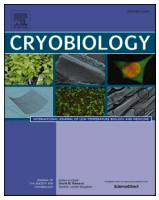

# Comparison of the recovery rate of vitrified hatched blastocysts and the frequency of blebbing between different thawing protocols

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ABSTRACT

This study evaluated the effects of different warming protocols on the recovery rate and blebbing frequency of vitrified hatched blastocysts, aiming to optimize embryo warming methods for improved clinical outcomes. A total of 650 patients (857 cycles) were included, with blastocysts warmed using either the conventional multi-step dilution method or a rapid warming method involving direct immersion in a low-concentration sucrose solution. Recovery rate, blebbing frequency, implantation rate, and clinical pregnancy rate were compared, along with the relationship to embryo morphology at the time of freezing. While recovery rates were similar between the two methods (conventional: 123.67 %, rapid: 124.45 %), the rapid method significantly reduced blebbing frequency (5.1 % vs. 10.9 %, $P < 0.05$). Implantation and clinical pregnancy rates were similar between the two groups. However, the rapid warming method effectively reduces blebbing while maintaining recovery rates. These findings suggest that rapid warming may improve embryo stability by reducing osmotic stress, supporting its potential clinical benefit and the need for further studies on long-term outcomes such as live birth rates.

## 1. Introduction

In recent assisted reproductive technologies (ART), vitrification has become the standard method for long-term embryo preservation [13,15,28]. Together with the improvement in blastocyst culture outcomes, it has contributed significantly to the success rates of frozen-thawed embryo transfer (FET) [1]. While factors such as equilibration during freezing [11], sucrose concentration, and external conditions are known to affect embryo viability, differences in warming protocols can also influence the viability and recovery of embryos after warming, which in turn can affect pregnancy outcomes after transfer [18]. In particular, the phenomenon of blebbing, a blister-like appearance caused by cell membrane swelling that occurs during embryo warming, has attracted attention as an indicator of cell membrane fragility and osmotic stress [24]. However, its impact on embryo recovery and implantation ability have not yet been fully investigated [8]. This study focuses on evaluating the effects of different warming protocols on embryo recovery and blebbing frequency of vitrified hatched blastocysts [6]. Two protocols were investigated: the conventional multi-step dilution protocol (conventional method) [16] and the rapid warming protocol (rapid method) in which embryos are directly placed in a low-concentration sucrose solution (0.25M sucrose) [2]. Observations were limited to hatched blastocysts that underwent shrinkage treatment and complete removal of zona pellucida at the time of freezing in order to ensure obtaining precise recovery rates through measuring embryo expansion [4], as well as to ensure accurate observation of blebbing phenomena. The evaluation includes the following aspects [17,30]:

The diameters of the blastocysts were measured 1 h after warming to assess their recovery rates, with previous studies suggesting that these recovery rates are correlated with embryo viability and developmental capacity [7,29]. In addition to measuring recovery, the presence of blebbing was recorded immediately after warming. The frequency of blebbing was compared between the two warming protocols, with prior research indicating that rapid osmotic changes are the primary cause of this phenomenon [5]. Furthermore, the impact of blebbing on pregnancy outcomes was evaluated by comparing the implantation rates, as indicated by serum hCG levels, of vitrified blastocysts transferred after thawing. Previous studies have suggested that blebbing may be associated with reduced pregnancy rates, even when embryos with blebbing appear to have recovered morphologically [27]. To better understand

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the potential impact, the recovery rates and frequency of blebbing were analysed in relation to the morphological characteristics of the embryos at the time of freezing. Post-transfer outcomes, including implantation (hCG-positive rate) and clinical pregnancy rates (gestational sac confirmation), were assessed to evaluate the clinical significance of blebbing suppression.

The purpose of this study is to optimize embryo warming protocols, based on the results of this study, thereby contributing to the improved success rates of frozen-thawed embryo transfers, as well as to provide foundational insights for the future improvement of warming protocols in ART.

## 2. Materials and methods

This study included 650 patients (857 cycles) who visited our clinic with infertility as their main complaint and were scheduled for single embryo transfer cycle of frozen-thawed blastocyst between June 2024 and January 2025. Hatched blastocysts that underwent vitrification were thawed using one of the two warming protocols: the conventional multi-step dilution protocol with sucrose solutions (1.0 → 0.75 → 0.5 → 0.25M; conventional method), and the rapid warming protocol involving direct immersion in a low-concentration sucrose solution (0.25M; rapid method). The frequency of blebbing, recovery rate of blastocysts, and post-transfer pregnancy outcomes were closely examined for each protocol (Fig. 1).

### 2.1. Selection of target embryos and vitrification method

The embryos used in this study were cultured in Human Tubal Fluid Medium (FUJIFILM Irvine Scientific, US, REF 90125) for days 1–2. Fertilized embryos were cultured to blastocyst stage on day 5 or day 6 using time-lapse culture (Vitrolife; Embryo Scope FLEX, Sweden) in ONESTEP medium (Naka medical, JAPAN, REF 08020) thereafter (37 °C in a humidified air of 5% $CO_2$, 5% $O_2$ and 90% $N_2$). Before vitrification, the blastocysts underwent shrinkage treatment followed by complete zona pellucida removal via laser (hatched blastocysts), and those with an inner cell mass (ICM) and trophectoderm (TE) grading based on the Gardner system that reached A or B, a maximum blastocyst diameter of ≥170 μm, and a TE cell count of ≥12 were selected for vitrification (Table 1). Those blastocysts were vitrified using a standard vitrification protocol with high-concentration sucrose solutions and dimethyl sulfoxide (DMSO).

**Table 1**
Details of vitrified blastocysts at the time of thawing by conventional/rapid methods.

| | Conventional method | Rapid method | P value |
|---|---|---|---|
| No. of vitrified blastocysts (pcs) | 700 | 157 | |
| Average age (yrs) | 37.73 ± 4.28 | 38.17 ± 4.06 | 0.241 |
| Developmental stage of embryos at the time of thawing: | | | |
| Day 5 embryos, % (pcs) | 51.6 (361) | 53.5 (84) | 0.662 |
| Day 6 embryos, % (pcs) | 48.4 (339) | 46.5 (73) | 0.662 |
| Mean blastocyst diameter (μm) | 206.52 ± 31.12 | 204.39 ± 28.34 | 0.898 |
| No. of trophectoderm cells (pcs) | 14.41 ± 2.10 | 14.15 ± 2.05 | 0.445 |
| Survival rate after thaw, % (pcs) | 98.6 (690) | 99.4 (156) | 0.686 |
| Implantation rate (HCG≧20mIU), % (pcs) | 56.8 (392) | 58.3 (91) | 0.729 |
| Clinical Pregnancy Rate (gestational sac confirmed), % (pcs) | 50.7 (350) | 50.6 (79) | 0.985 |

Data are expressed as mean ± SD or n/N (%).
Means were compared using the *t*-test with ANOVA and rates were compared using the chi-square test.

### 2.2. Comparison of warming protocols

Warming was performed using one of the following two protocols.

- **Conventional method (Multi-step dilution protocol from high-concentration sucrose solution to low concentration sucrose solution):** Blastocysts were placed directly into the warming solution (TS; 1.0M sucrose, 1 min) prepared in our laboratory, followed by sequential immersion into the dilution solutions with different concentrations of sucrose which were also prepared in our laboratory (DS; 0.75 → 0.5 → 0.25M sucrose, 3 min each, 37 °C). After the thawing, blastocysts were cultured for recovery in EmbryoNida medium (Kitazato Medical Supply, JAPAN, Catalog REF 93721) which had been prepared from the day before, until the time of transfer.
- **Rapid method (Simple dilution to low-concentration sucrose solution):** Blastocysts were placed directly in Ultra RapidWarm Blast medium (Vitrolife, Sweden, REF 10150, 0.25M sucrose) for 2 min at 37 °C. Post-warm culture for recovery was conducted using Embryo Nida medium until transfer, in the same manner as the conventional method.

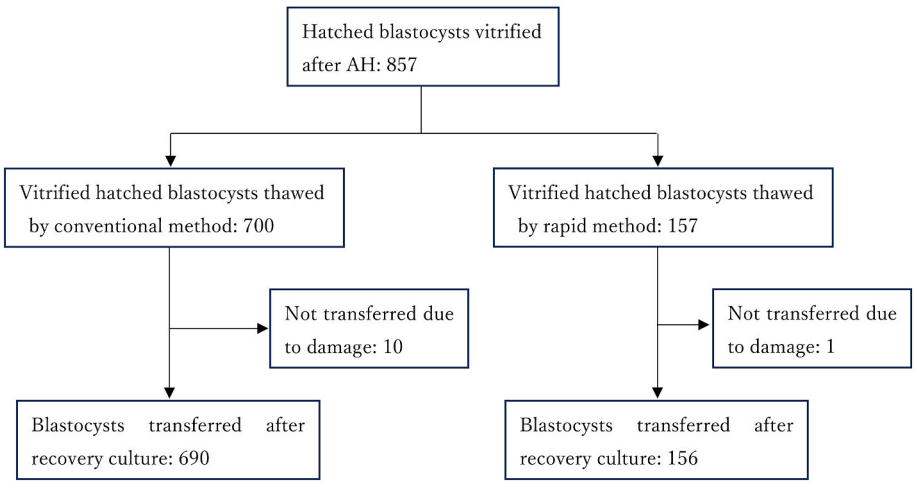



**Fig. 1.** Flowchart of this study.

### 2.3. Evaluation of recovery rate and blebbing frequency

Under inverted microscopy (×200), the recovery rate was measured as the mean diameter of blastocysts (average of the longest and shortest diameters) immediately after and 1 h after the warming, and the presence or absence of the blebbing was observed immediately after the warming (Fig. 2).

- **Recovery Rate (Blastocyst Expansion):** Mean blastocyst diameter change at 1-h post-warm was measured using digital image analysis, with the pre-warm diameter as the baseline, and compared between the two methods.
- **Blebbing Frequency:** Presence or absence of blebbing was observed immediately after the warming as an indicator of trophectoderm membrane stress and the results were compared between the two methods.

### 2.4. Evaluation of embryo transfer and pregnancy outcomes

The following factors were compared between the two methods for cases in which embryo transfer was performed after the warming.

- **Implantation Rate:** Positive serum hCG levels (≥20 mIU) on day 10 of the transfer were used to confirm the embryo's implantation ability.
- **Clinical Pregnancy Rate:** Gestational sac confirmation via ultrasound at 5 weeks of pregnancy was used to assess pregnancy outcomes.

### 2.5. Analysis of the causes of blebbing

To determine the frequency of blebbing and its causes, the odds ratios of blebbing were analysed in relation to the morphological characteristics of the embryos at the time of vitrification, such as mean blastocyst diameter and TE cell count.

### 2.6. Statistical analysis

For patient characteristics, summary statistics were constructed using frequency and proportion of categorical data, and means, standard deviations (SDs), and range for continuous variables. Normality of continuous variables was assessed using the Shapiro–Wilk test to determine the suitability for parametric testing. Significant differences between parameters were statistically analysed using t-tests or chi-square tests with ANOVA, and P values ≤ 0.05 were considered statistically significant. Adjusted odds ratios were analysed by logistic regression analysis using multivariate analysis, and P values ≤ 0.05 were considered statistically significant.

## 3. Results

Among vitrified blastocysts, 700 embryos were thawed using the conventional multi-step dilution method and 157 using the rapid warming method. The number of embryos deemed unsuitable for transfer due to severe damage during warming was 10 for the conventional method and 1 for the rapid method. This suggests a notable difference in embryo survival between the two methods, with the rapid method demonstrating a significantly lower incidence of damage. The recovery rate, defined as the expansion of blastocyst diameter from immediate post-warm to 1 h later, was 123.67 ± 13.18 % for the conventional method and 124.45 ± 13.58 % for the rapid method. This suggests a notable difference in embryo survival between the two methods, with the rapid method demonstrating a significantly lower incidence of damage. The recovery rate, defined as the expansion of blastocyst diameter from immediately post-warming to 1 h later, was 123.67 ± 13.18 % for the conventional method and 124.45 ± 13.58 % for the rapid method. There was no significant difference between the two groups (P = 0.502), indicating that both methods resulted in similar blastocyst recovery post-warm.

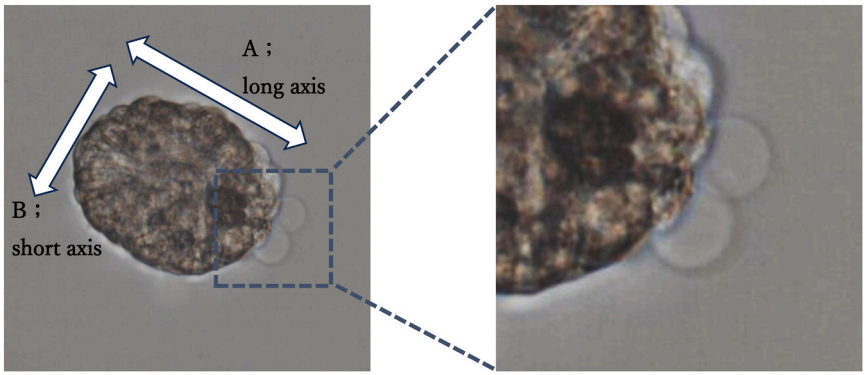
However, a significant difference was observed in the occurrence of blebbing. The conventional method had a blebbing frequency of 10.9 % (75/690), whereas the rapid method showed a significantly lower blebbing frequency of 5.1 % (8/156) (P < 0.05), suggesting that the rapid warming method may be associated with reduced cellular stress during the thawing process (Table 2).

A comparison of pregnancy outcomes post-transfer revealed that the

**Table 2**
Rates of blastocyst recovery and blebbing frequency at the time of thawing by conventional and rapid methods.

| | Conventional method (n = 690) | Rapid method (n = 156) | P-value |
|---|---|---|---|
| Mean blastocyst diameter: | | | |
| Immediately after thawing (μm) | 113.33 ± 11.70 | 113.40 ± 12.23 | 0.943 |
| 1 h after thawing (μm) | 134.01 ± 17.20 | 135.51 ± 17.61 | 0.326 |
| Recovery rate (%) | 123.67 ± 13.18 | 124.45 ± 13.58 | 0.502 |
| Blebbing incidence, % (n) | 10.9 (75) | 5.1 (8) | <0.05 |

Data are expressed as mean ± SD or n/N (%).
Means were compared using the *t*-test with ANOVA and rates were compared using the chi-square test.
Results with P < 0.05 were deemed statistically significant.




Mean blastocyst diameter (μm) = (A+B)/2 Example of blebbing observed immediately after thawing

**Fig. 2.** Mean blastocyst diameter measurement and blebbing observation.
Mean blastocyst diameter (μm) = (A + B)/2 Example of blebbing observed immediately after thawing

HCG-positive implantation rate was 56.8 % (392/690) for the conventional method and 58.3 % (91/156) for the rapid method, showing no significant difference (P = 0.729). Similarly, the clinical pregnancy rate was 50.7 % (350/690) for the conventional method and 50.6 % (79/156) for the rapid method, with no significant difference (P = 0.985) (Table 1). These results indicate that while the rapid method reduces blebbing, it does not lead to a statistically significant improvement in implantation or clinical pregnancy rates compared to the conventional method.

Factors contributing to blebbing occurrence (mean blastocyst diameter and TE cell count) were examined by logistic regression analysis for both methods. Adjusted odds ratios for blebbing (considered positive) were 1.022 (95 % CL: 1.013–1.030, P < 0.001) for mean blastocyst diameter and 0.901 (95 % CL: 0.812–0.994, P < 0.05) for TE cell count in the conventional method. For the rapid method, the adjusted odds ratios were 1.012 (95 % CL: 0.984–1.038, P = 0.352) for mean blastocyst diameter and 0.892 (95 % CL: 0.631–1.176, P = 0.438) for TE cell count. Blebbing frequency in the conventional method increased with larger blastocyst diameter (P < 0.001) and decreased with higher TE cell count (P < 0.05). However, no significant correlations were observed between blebbing frequency and either blastocyst diameter or TE cell count in the rapid method (N.S) (Table 3).

## 4. Discussion

This study compared two warming protocols— the conventional multi-step dilution method and the rapid method utilizing simple dilution— and found no significant difference in recovery rates between the two groups [19,20,23,25]. This suggests that the basic recovery ability of embryos is comparable between the two protocols [22]. However, an important distinction emerged: the frequency of blebbing was significantly lower with the rapid method compared to the conventional method.

The suppression of blebbing observed in the rapid method can be attributed to a substantial reduction in the embryos' exposure time to sucrose, leading to decreased osmotic stress. By placing embryos directly into a 0.25M sucrose solution, the rapid method shortens the warming process compared to the conventional method. This allows for faster completion of osmotic changes, potentially mitigating adverse effects in a shorter timeframe. Although the multi-step dilution in the conventional method was intended to minimize cell stress by avoiding abrupt osmotic changes, it is noteworthy that the results suggest rapid warming alleviates stress on the blastocyst cell membrane more effectively. Given that blebbing is a phenomenon reactive to osmotic stress immediately after warming, the rapid method's stress reduction likely contributed to lower blebbing rates.

Interestingly, despite reduced blebbing frequency, no significant difference in recovery rates was observed. This implies that blebbing may not directly impact the initial recovery capacity of warmed blastocysts [9].

**Table 3**
Factors associated with blebbing in conventional and rapid methods.

| | | Multivariate analysis | |
|---|---|---|---|
| | Adjusted odds ratio | 95 % confidence interval | P-value |
| Conventional method: | | | |
| Mean blastocyst diameter (μm) | 1.022 | 1.013-1.030 | <0.0001 |
| No. of TEs (pcs) | 0.901 | 0.812-0.994 | <0.05 |
| Rapid method: | | | |
| Mean blastocyst diameter (μm) | 1.012 | 0.984-1.038 | 0.352 |
| No. of TEs (pcs) | 0.892 | 0.631-1.176 | 0.438 |

Results with P < 0.05 were deemed statistically significant.
TEs: trophectoderm cells.

Implantation and clinical pregnancy rates also showed no significant differences between the two groups. This suggests that factors influencing post-transfer pregnancy outcomes are more complex and not solely determined by recovery capacity or blebbing frequency. However, suppressing blebbing could contribute to embryo stability after transfer, potentially influencing pregnancy outcomes positively, and this warrants further investigation.

From a morphological perspective, regarding the relationship between the mean blastocyst diameter and number of TE cells around the perimeter of the blastocyst and the blebbing frequency, a larger mean blastocyst diameter correlated with a significantly higher frequency of blebbing during warming with the conventional method. It can be inferred that blastocysts with a larger mean diameter, i.e. with relatively large TE cells, are more susceptible to osmotic pressure changes during warming due to the generally larger and more extended cell membrane area, and are therefore more prone to blebbing. In particular, in the conventional method, stepwise dilution proceeds more slowly and for a longer period of time than in the rapid method, which increases the pressure on the cell membrane and may lead to an increase in the frequency of blebbing. In comparison, the rapid method requires only a short time for the osmotic pressure change regardless of the blastocyst size, thus reducing the stress on the cell membrane, which may have suppressed the frequency of blebbing occurrence.

In the conventional method, embryos with a higher number of TE cells demonstrated significantly lower blebbing frequency. This may be due to the fact that blastocysts with high TE cell counts are high-quality embryos with a relatively stable structure, and thus the occurrence of blebbing at warming may be reduced. Conversely, in blastocysts with fewer TE cell counts, the stability of the cell membrane structure is reduced, and particularly in larger blastocysts with fewer TE cell counts, the thinning portions of the stretched cell membrane are vulnerable. Those may contribute to the increased frequency of blebbing in the conventional warming method in which the blastocysts experience relatively prolonged hyperosmotic condition.

Overall, while no significant difference in recovery rates was noted, the rapid method was observed to suppress blebbing significantly. As blebbing reflects membrane stress and can potentially impact embryo stability and subsequent development [12], suppressing it via the rapid method appears beneficial for an embryo's physiological recovery [10]. In particular, as the rapid method has been suggested to mitigate the impact of blastocyst growth rate and blastocele size on blebbing development, this study has shown this aspect of the rapid method is likely to contribute to improved embryonic stability [26]. Moreover, reports in other animal species have shown that simplified the rapid warming techniques yield comparable results to the conventional multi-step dilution method, demonstrating that they are not only effective in preserving embryo viability but also feasible for practical use in reproductive programs [3].

One major advantage of transitioning to the rapid method is the shorter warming duration. Notably, with this study, even for some embryos with compromised TE morphology, the rapid method was shown to reduce TE cell damage compared to the conventional method, while maintaining comparable clinical pregnancy outcomes. Future studies targeting cases through to childbirth are essential to elucidate the effects of the rapid warming method and the mechanisms underlying the blebbing development [14,21].

## CRediT authorship contribution statement

**Tsuyoshi Okubo:** Writing – original draft. **Ai Higuchi:** Project administration, Investigation, Data curation. **Kenta Higuchi:** Project administration, Investigation. **Tomomi Taguchi:** Project administration, Investigation. **Ryoko Matsuo:** Project administration, Investigation. **Noriyuki Onda:** Project administration, Investigation. **Teruaki Hayashi:** Supervision. **Kenji Omi:** Project administration, Investigation, Conceptualization. **Tomoya Segawa:** Writing – review & editing,

Supervision, Project administration, Investigation.

## Human rights statements 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.

## Approval by ethics committee

This study was conducted after approval by Shinbashi yume clinic Ethics Committee (approval number: 2024-02). Written informed consent was obtained from all patients for being included in the study.

## Clinical trial registration details

This study has not been registered.

## Conflict of interest

None of the authors of this article has a financial or personal relationship with other people or organizations that could inappropriately influence of the paper.

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