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Partial Dehydration Enables Low-Cryoprotectant Vitrification of Human Embryos

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Abstract

Research question

Can controlled partial dehydration enable successful vitrification of human embryos using only equilibration solution, thereby reducing cryoprotectant concentration exposure?

Design

A total of 212 day-2 human embryos derived from abnormal fertilization (1PN and 3PN) following intracytoplasmic sperm injection (ICSI) were vitrified using either a conventional vitrification protocol or an equilibration solution (ES) alone, combined with controlled partial dehydration (~26% droplet water loss) and a minimal droplet volume (≤1 µL) before immersion in LN.

Results

Partial dehydration increased the effective cryoprotectant concentration to approximately 19.3%, enabling successful vitrification with substantially lower cryoprotectant exposure than conventional vitrification solutions (30%). Embryos vitrified using ES alone following partial dehydration exhibited survival rates (93%) and blastocyst formation rates (22%) comparable to those achieved using conventional vitrification (89% and 24%, respectively). Successful low-CP vitrification required high cooling rates (>20,000 °C/min), achieved using low thermal-mass cryo-carriers.

Conclusions

These findings demonstrate that controlled partial dehydration can reduce cryoprotectant requirements while maintaining embryo developmental competence. This study confirms that under low-cryoprotectant vitrification conditions, droplet volume and cooling rate are important factors for successful vitrification of embryos. This approach may simplify and shorten cryopreservation procedures, reducing cryoprotectant-associated toxicity and may facilitate the development of automated vitrification systems in clinical IVF laboratories.

Keywords: vitrification; partial dehydration; cryoprotectant toxicity; human embryos

Introduction

Vitrification is a physical process in which an aqueous solution transitions into an amorphous glassy state without ice crystal formation, thereby avoiding the mechanical and osmotic damage associated with conventional freezing and thawing (Arav, 2002; Kuwayama, 2005). In assisted reproductive technology (ART), cryopreservation of gametes and embryos is routinely performed using either slow freezing or vitrification (Saragusty and Arav, 2011).

Slow freezing employs relatively low concentrations of cryoprotectants (CPs) but is associated with ice crystal formation, chilling injury, and reduced post-thaw viability. In contrast, vitrification minimizes ice crystallization through rapid cooling but requires exposure to high CP concentrations, which may induce osmotic stress and chemical toxicity. Increasing evidence suggests that cryoprotectants can influence cellular physiology and developmental competence, particularly in sensitive reproductive cells.

The probability of successful vitrification is determined by three interdependent parameters: cooling and warming rates, solution viscosity, and sample volume. Increasing cooling rates and viscosity while minimizing sample volume markedly improves vitrification success. The development of minimal-drop-size (MDS) approaches (Arav, 2014; Kuwayama, 2007) enabled routine vitrification of human oocytes and embryos. However, current protocols remain technically demanding and typically require CP concentrations exceeding 30% (v/v). Exposure to such concentrations has been associated with alterations in gene expression, DNA methylation, and epigenetic regulation in gametes and early embryos (Petrussa et al., 2014).

Partial dehydration before cooling offers a novel potential strategy to address both the biological and logistical limitations of current cryopreservation methods. Controlled water removal increases solution viscosity and reduces freezable water content, thereby lowering the cryoprotectant concentration required for vitrification and potentially reducing osmotic and toxic injury. In embryos, partial dehydration may permit successful vitrification using equilibration solution alone when combined with sufficiently high cooling rates.

In the present study, we evaluated the feasibility and performance of partial dehydration as a unified strategy using abnormally fertilized human embryos. We assessed the survival and developmental competence of day-2 human embryos (3PN, 1PN) vitrified using equilibration solution (ES) alone, in minimal volumes following controlled partial dehydration.

Materials and Methods

Partial Dehydration and Vitrification of Human Embryos

Ethics approval

The study entitled "Freeze drying of abnormal fertilized human embryos (1PN and 3PN).Was approved by the institutional review board (IRB) of Shamir Hospital () This study was conducted in accordance with the principles of the declaration of Helsinki and its subsequent amendments.

Embryo Source and Selection

Day-2 embryos derived from intracytoplasmic sperm injection (ICSI) cycles exhibiting abnormal fertilization, characterized by the presence of either one pronucleus (1PN) or three pronuclei (3PN), were included in the study. Embryos containing at least two blastomeres and exhibiting ≤50% fragmentation were considered suitable for inclusion.

A total of 212 embryos obtained from 127 ICSI cycles were randomly allocated to experimental and control groups. Allocation was performed without consideration of embryo morphology, blastomere number, patient age, or post-vitrification storage duration to minimize selection bias and ensure comparable distribution of embryo characteristics among groups.

Equilibration Solution and Vitrification Protocol

The equilibration solution (ES) consisted of 7.5% (v/v) dimethyl sulfoxide (DMSO) and 7.5% (v/v) ethylene glycol prepared in TCM supplemented with 20% serum substitute supplement (SSS) (CooperSurgical, Inc., CT, USA).

Embryos were assigned to one of three groups:

- **Group A (Control):** Conventional vitrification using a commercial vitrification kit and either CryoTop (Origio, Sage) or CRYOLOCK™ (Biotech Inc. by Irvine Scientific) carriers.
- **Group B:** Exposure to ES only, loading in a minimal volume (<1 µL; droplet diameter approximately 1.5 mm), followed by partial dehydration for 30 s and vitrification using a CryoTop carrier.
- **Group C:** Identical protocol to Group B, except that vitrification was performed using a CRYOLOCK™ carrier.

Partial Dehydration Procedure

Drops were formed using a calibrated pipet (P2 Gilson WI, USA) (<1 µL; diameter 1.5 mm) then exposed to laminar airflow (150 Pa, 0.5 m/s) at 28°C for 30 seconds, to ensure controlled dehydration. The dehydration rate was dependent on temperature (T=28°C), airflow rate (F= 0.5m/sec), and droplet surface area (S= 3.53mm^2). Droplets weight was measured before and after 30s of exposure to laminar airflow, and dehydration percentage was calculated gravimetrically using 100 droplets of ES (1 µL each) exposed under identical conditions. Droplet weight (all 100 drops) was measured before and after dehydration, and the percentage of water loss was calculated from the reduction in droplet mass. The gravimetric measurements were intended to quantify water loss from the extracellular microdroplet environment rather than directly measure intracellular water loss from embryos. Therefore, the calculated dehydration percentage represents droplet dehydration and serves as an indirect indicator of the physicochemical changes experienced by embryos during vitrification.

Warming and Embryo Culture

Embryos were warmed according to the manufacturer's instructions for the vitrification system used (Origio-Medicult). Post-warming survival was assessed immediately after warming. Embryos were considered viable if at least 50% of the blastomeres survived.

Surviving embryos were cultured under standard laboratory conditions using GTL medium (Vitrolife, Sweden) until the blastocyst stage. Blastocyst formation rate was used as an indicator of post-warming developmental competence.

Cooling Rate Measurements

Cooling rates were evaluated using two independent methods. First, cooling performance was estimated acoustically by measuring the duration of liquid nitrogen boiling following immersion of the carrier. A smartphone (Galaxi 4, Samsung, South Korea) was used to detect the length (seconds) and the peak (dB) produced by the sound of boiling LN. The sound detector was downloaded from the App store (Sound Meter). We also compared the cooling rates of the two carriers by inserting a fine (50 µm) T type thermocouple connected to a data logger (Almemo® 2290-4, MRC Ltd, Holon, Israel) the thermocouple positioned on either the CryoTop or CRYOLOCK™ carrier during immersion in liquid nitrogen.

Cryomicroscopy Evaluation of Vitrification

The vitrification properties of the equilibration solution before and after partial dehydration were evaluated by cryo-stereomicroscopy. Drops of ES (1 µL) were placed on a glass coverslip and either subjected to partial dehydration or left untreated. The droplets were then plunged directly into liquid nitrogen and examined using a cryo-stereomicroscope.

Glass formation was assessed based on droplet appearance. Transparent droplets were classified as vitrified, whereas opaque droplets containing visible ice crystals were classified as frozen.

Statistical Analysis

Data are presented as mean ± standard deviation (SD) or percentage (n). Normality was assessed before application of parametric tests. Continuous variables were compared using Student's t-test, while categorical outcomes including embryo survival and blastocyst formation rates were compared using χ^2 or Fisher's exact test, as appropriate. Statistical significance was defined as $p<0.05$.

The primary study endpoints were post-warming embryo survival and blastocyst formation rates. Given the exploratory proof-of-concept nature of the study and the limited availability of abnormal human embryos, no formal sample-size calculation was performed.

Results

Partial Dehydration of Equilibration Solution

Based on measurements of 100 droplets exposed under identical conditions, water loss averaged 26%. Assuming negligible evaporation of DMSO and ethylene glycol during the 30-second dehydration period, the measured reduction in droplet mass corresponds to an estimated increase in cryoprotectant concentration from 15% to approximately 19.3%. This value represents a calculated effective concentration rather than a direct analytical measurement. This concentration is substantially lower than that used in the conventional vitrification solution (VS=30% cryoprotectants).

Cryomicroscopy Assessment of Glass Formation

Cryomicroscopic evaluation demonstrated that all partially dehydrated droplets with 19.3% CPs appeared transparent (Figure 2) immediately after immersion in liquid nitrogen, indicating successful vitrification. In contrast, most non-dehydrated droplets appeared opaque and exhibited visible ice crystal formation (Figure 1), indicating freezing rather than vitrification. In addition, droplets containing 19.3% CPs cooled using CryoTop did not vitrify (Data not shown).

Embryonic development

In total, 212 day-2, 1PN and 3PN embryos were collected and allocated in the three experimental groups: 84 (group A) , 88 (group B) and 40 (group C). Mean patients' age was similar across groups 33.1±6.2 years (group A), 32.5±6.1 and 31.8±5.7 years (group B and C, respectively). The mean number of blastomeres was also similar, 4.9±2.1, 4.7±1.6 and 4.9±2.3, respectively. Mean number of abnormal fertilizations (1PN and 3PN) per patient was 2.2±1.8 in groups A and B and 2.5±2 in group C. Interval storage time in LN was 24.1±30.1,

19.3±42.3, and 28.6±27.8 days, respectively. Embryos vitrified using equilibration solution alone following partial dehydration (Group B) exhibited survival and blastocyst formation rates comparable to those achieved with conventional vitrification (Group A). In group A there was no difference in embryo survival using CryoTop or CRYOLOCK™. Survival rates (table 1) were 93% and 89% in Groups B and A, respectively, while blastocyst formation rates were 22% and 24%, respectively (p=ns).

In contrast, embryos vitrified using the same low-cryoprotectant protocol on CRYOLOCK™ carriers (Group C) demonstrated significantly reduced survival to 27.5% and failed to develop to the blastocyst stage

Cooling Rate Measurements

Cooling rates were assessed using both acoustic measurements of liquid nitrogen boiling and direct thermocouple recordings obtained with a 50-µm T-type thermocouple positioned on the carrier surface. The boiling time of CryoTop was 1.1 seconds with a peak noise of 10dB (Figure 3) while CRYOLOCK boiling time was 1.8 seconds with more than 20dB (Figure 4). CryoTop carriers achieved cooling rates exceeding 29,200±721°C/min and exhibited a significantly shorter liquid nitrogen boiling period following immersion (Figure 2), indicative of more efficient heat transfer. In contrast, CRYOLOCK™ carriers achieved cooling rates of 18,610± 512°C/min and displayed a prolonged boiling period (Figure 3). Under low-cryoprotectant vitrification conditions, the reduced cooling rate of CRYOLOCK™ was associated with inferior embryo survival and the absence of blastocyst development, highlighting the critical role of rapid cooling in achieving successful vitrification.

Discussion

Partial dehydration prior to cooling appears to be a practical strategy for simplifying vitrification safety while reducing cryoprotectant exposure in human embryos. Under conventional vitrification conditions, the higher cryoprotectant concentration likely provides a wider safety margin against modest reductions in cooling rate. In contrast, under reduced-cryoprotectant conditions, vitrification becomes increasingly dependent on rapid heat transfer, rendering carrier-dependent cooling differences biologically relevant. By reducing freezable water content and increasing solution viscosity, controlled water removal may facilitate vitrification at lower cryoprotectant (CP) concentrations while limiting osmotic stress and chemical exposure (Gosden, 2011; Vajta & Kuwayama, 2007; Cobo & Díaz, 2011).

In this proof of concept experimental study, successful embryo vitrification was achieved using equilibration solution alone when partial dehydration (~26%), minimal sample volume, and rapid cooling rates were combined. This level of dehydration likely contributed to increased solute concentration and reduced droplet volume, both of which are known to favor vitrification (Arav, 2002; Saragusty & Arav, 2011; Arav A. 2014). To our knowledge, this is the first study investigating controlled partial dehydration as a strategy for low-cryoprotectant vitrification of human embryos.

To further examine whether the increased cryoprotectant concentration of Equilibration Solution (ES) alone (from 15% to 19.3%) could account for the observed vitrification, we performed an additional control experiment using 1 µL droplets containing 19.3% cryoprotectants, corresponding to the estimated concentration reached after partial dehydration (Unpublished experiment). These droplets crystallized upon cooling and did not vitrify. In contrast, partially dehydrated droplets, which reached a similar cryoprotectant

concentration (~19.3%), remained vitrified. This observation indicates that vitrification was not achieved solely as a consequence of increased cryoprotectant concentration. Rather, the approximately 26% reduction (to 0.74 µL) in droplet volume produced by controlled dehydration also contributed to suppressing ice formation and promoting vitrification. Overall these findings support the hypothesis that all three factors; **concentration of CPs, volume and cooling rate** play important roles in the improved vitrification outcome observed following partial dehydration.

Embryo survival and subsequent blastocyst development under these conditions were comparable to those obtained using conventional high-CP vitrification protocols, suggesting that partial dehydration may partially compensate for reduced CP concentration under appropriately controlled physical conditions (Kuwayama, 2007; Cobo et al., 2008; Levi-Setti et al., 2016).

Differences in performance between Cryotop and CRYOLOCK™ carriers further highlight the importance of cooling dynamics in low-CP vitrification. Cryotop achieved higher cooling rates than CRYOLOCK™ under identical experimental conditions, likely reflecting differences in thermal mass and heat exchange efficiency (Casillas et al., 2014). These findings are consistent with the concept that carrier-dependent heat transfer limitations, including vapor layer formation around the device, may influence effective cooling rates (Fahy & Wowk, 2015; Casillas et al., 2014). Importantly, such differences became functionally relevant only under reduced-CP conditions, whereas both systems performed similarly under standard vitrification protocols.

The increased sensitivity to cooling rate observed in low-CP conditions underscores the interdependence of physical parameters governing vitrification success, including cooling rate, sample volume, viscosity, and solute concentration (Arav 2002, Fahy & Wowk, 2015; Kopeika et al., 2015). When CP concentrations are reduced, vitrification appears more dependent on achieving sufficiently rapid heat transfer, whereas conventional high-CP protocols may buffer against such limitations.

Beyond immediate developmental outcomes, reducing exposure to high CP concentrations may have potential biological advantages, as osmotic stress and CP toxicity have been associated with alterations in cellular and molecular parameters in gametes and embryos (Kopeika et al., 2015; Wang et al., 2010; Petrussa et al., 2014). Although molecular endpoints were not assessed in this study, the comparable developmental competence observed following partial dehydration supports further investigation into whether reduced-CP approaches may offer safety benefits at the cellular or epigenetic level (Fuller, 2004).

A limitation of this study is the use of embryos derived from abnormal fertilization (1PN and 3PN). Although Ahlström et al. 2026, reported that blastocyst-stage 1PN embryos can achieve live birth rates comparable to those of normally fertilized embryos, our findings should still be validated in euploid embryos derived from normal fertilization before clinical translation.

In conclusion, partial dehydration represents a promising adjunct to conventional vitrification strategies by enabling reduced CP exposure while maintaining developmental competence under optimized physical conditions. These findings support further refinement of low-CP vitrification approaches and highlight the importance of integrating thermophysical principles into cryopreservation design for assisted reproductive technology

(Gosden, 2011; Vajta & Kuwayama, 2006; Cobo & Díaz, 2011). These findings provide proof of concept for a simplified vitrification strategy; the proposed approach offers several practical advantages over conventional vitrification protocols. First, the procedure is simpler because it eliminates the final exposure step to the vitrification solution (VS), thereby shortening the protocol and reducing handling time. Second, it removes the technically demanding step of producing extremely small volume onto the cryocarrier, which is one of the most critical operator-dependent aspects of current vitrification methods. Finally, by simplifying the workflow and reducing manual manipulation, this approach may facilitate the development of automated vitrification systems, improving standardization, reproducibility, and ease of implementation in clinical IVF laboratories.

AI use disclosure: Artificial intelligence (AI) was used solely to assist with language editing, grammar, spelling, and improving the clarity of the text. All scientific content, study design, analyses, interpretations, and conclusions were developed and verified by the authors.

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Figure 1. Minimal drop size (MDS- <1ul) equilibration solution droplets following direct immersion in liquid nitrogen, showing ice crystallization (dark drops) in the absence of partial dehydration.

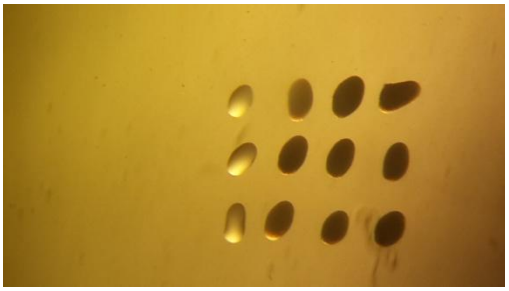


Figure 2. Equilibration solution droplets (1ul) following 30 s of partial dehydration before immersion in liquid nitrogen, demonstrating successful vitrification without ice crystallization.

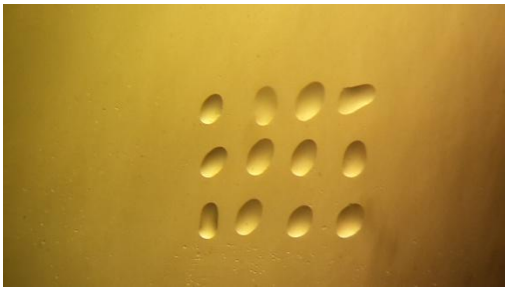


Figure 3. Acoustic profile (left) of the CryoTop carrier during immersion in liquid nitrogen, showing a shorter boiling duration and lower acoustic intensity, consistent with reduced thermal mass.

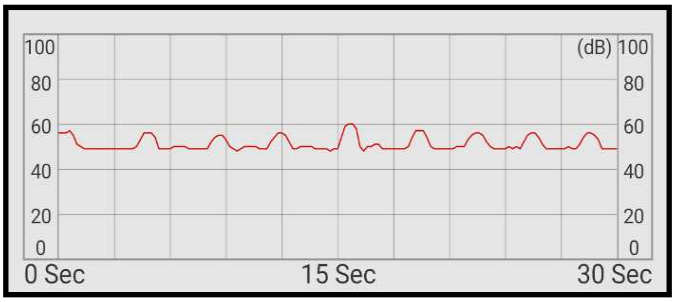


Figure 4. Acoustic profile (right) of the CRYOLOCK™ carrier during immersion in liquid nitrogen, showing prolonged boiling and higher acoustic intensity compared with the CryoTop carrier.



Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- table1.docx