ATATÜRK ÜNİVERSİTESİ YAYINLARI
ATATURK UNIVERSITY PUBLICATIONS

Research Article DOI: 10.62425/jlasp.1760612

Morphological and Mitochondrial Advantages of Ultrafast Freeze–Thaw in Oocyte Cryopreservation

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ABSTRACT

Cryopreservation of oocytes is central to assisted reproductive technologies (ART) and fertility preservation. Conventional vitrification (CVT), though widely used, entails prolonged exposure to high concentrations of cryoprotective agents (CPAs), which can induce cellular stress. Ultrafast freeze–thaw (UFFT) aims to minimize CPA exposure and achieve extreme cooling/warming rates, reducing cryodamage. To compare UFFT and CVT in mouse oocytes, focusing on spindle/polar body morphology, necrosis percentage via PI^+ extracellular vesicles (EVs), and mitochondrial unfolded protein response (mtUPR) activation. Metaphase II oocytes (n = 100/group) were assigned to fresh control, CVT, or UFFT groups. Post-thaw morphology was assessed via light/polarized microscopy; necrosis was quantified as PI^+ EV fraction by flow cytometry; mtUPR activation (Hsp60, Lonp1, Atf5, ClpP) was measured by qRT-PCR. Data were analyzed by ANOVA with Tukey's post-hoc test. UFFT oocytes exhibited significantly higher spindle integrity (91% ± 3%) and polar body retention (94% ± 3%) than CVT (65% ± 5% and 70% ± 4%, respectively; $p < .001$). PI^+ EV percentage was reduced in UFFT (18.0% ± 3.0%) compared to CVT (34.0% ± 4.0%; $p < .001$). mtUPR gene expression was highest in CVT, intermediate in UFFT, and lowest in the fresh control. Differences between UFFT and control were not statistically significant ($p > .05$). UFFT preserved oocyte morphology and reduced membrane damage compared to CVT, without significantly elevating mitochondrial stress markers above control. These findings support UFFT as a promising alternative in ART and warrant further large-scale and clinical studies.

Keywords: MT-UPR, Oocyte, Vitrification.

Received Date :07.08.2025
Revision Requested Date :28.11.2025
Last Revision Date :25.12.2025
Accepted Date :01.01.2026
Publication Date :12.02.2026

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Cite this article: Başar, M. (2026). Morphological and Mitochondrial Advantages of Ultrafast Freeze–Thaw in Oocyte Cryopreservation. *Journal of Laboratory Animal Science and Practices, 6*(1), 9-14. https://doi.org/10.62425/jlasp.1760612

Introduction

Oocyte cryopreservation is a critical component of reproductive medicine, underpinning in vitro fertilization (IVF) programs, fertility preservation for oncology patients, and genetic resource banking in animal breeding. The introduction of vitrification has markedly improved post-thaw oocyte survival and fertilization rates compared to conventional slow-freezing, primarily by preventing intracellular ice formation through ultra-rapid cooling in high concentrations of CPAs (Cha et al., 2011; Cobo et al., 2021; Cobo et al., 2020). However, CPA exposure imposes osmotic and chemical stresses that can compromise plasma membrane integrity, impair organelle function, and destabilize chromosomal architecture (Ahmadkhani et al., 2025; Gallardo et al., 2019; Martinez-Rodero et al., 2025).

Mitochondria, essential for meiotic spindle assembly and early embryonic development, are particularly vulnerable to cryoinjury (Galeska et al., 2025; May-Panloup et al., 2016; Van Blerkom, 2011). Mitochondrial stress can activate the mtUPR, a protective pathway involving the upregulation of chaperones and proteases such as Hsp60, Lonp1, Atf5, and ClpP (Dodge et al., 2024). While transient mtUPR activation may facilitate repair, excessive or prolonged activation has been linked to diminished oocyte developmental competence and impaired blastocyst formation (Babayev & Seli, 2015; Ergun et al., 2024; Yildirim et al., 2022).

Emerging evidence indicates that EVs released by oocytes and cumulus cells serve as noninvasive biomarkers of cellular health. EVs released by oocytes and cumulus cells function as a "liquid biopsy," providing a non-invasive, real-time indicator of cellular health. The composition of these EVs reflects cellular integrity: PI-positive EVs indicate membrane damage and necrotic debris, whereas healthy cells release EVs that contain beneficial microRNAs and proteins for maturation and fertilization. The quantity of EVs, particularly PI+ EVs, directly correlates with stress levels—higher counts signal cellular damage and predict reduced developmental competence. In comparison, lower counts indicate robust membrane integrity and better pregnancy outcomes. This measurement system enables researchers to quantify cryoinjury and assess oocyte viability using flow cytometry. (Berkay Akcay, 2023; Chen et al., 2022; Pallinger et al., 2017).

UFFT protocols, which achieve cooling rates of approximately 10^5 °C/min with minimal CPA exposure, have been proposed to mitigate osmotic and chemical damage relative to conventional vitrification (Cho et al., 2024; Liebermann et al., 2024; Schiewe et al., 2024; Wozniak et al., 2024). While UFFT has shown promise in embryos and certain gamete types, comparative studies against widely used clinical vitrification systems in murine oocytes remain limited.

We hypothesized that UFFT would (i) better preserve spindle and polar body morphology, (ii) reduce necrotic damage as indicated by PI^+ EV release, and (iii) attenuate stress-induced mtUPR activation relative to conventional vitrification.

Methods

C57BL/6 female mice were maintained according to Yale University's requirements for animal research, and all procedures were approved by the Institutional Animal Care and Use Committee (Date: March 14 2022, protocol no. 2022-11300).

Oocyte Collection and Experimental Groups

Mouse metaphase II (MII) oocytes were collected using standard protocols under the guidelines approved by the Yale Institutional Animal Care and Use Committee.

In short, 5-week-old C57BL/6 female mice (Charles River Labs) were super-ovulated by intraperitoneal (IP) injection of 5 international units (IU) of pregnant mare serum gonadotropin (PMSG; Folligon, Sigma-Aldrich). An additional injection of 5 IU of hCG (Chorulon, Sigma-Aldrich) was given 48 hours after the PMSG injection. MII oocytes were collected from the oviducts at 14 hours post-hCG injection and denuded enzymatically.

MII oocytes were allocated into:
Control: Fresh, unfrozen (n = 100)
CVT: Conventional vitrification using Kitazato kit (n = 100)
UFFT: Ultrafast freeze–thaw using Kitazato kit (n = 100)

Cryopreservation Protocols

Conventional oocyte vitrification was performed using Kitazato vitrification solutions (10-minute equilibration interval) on open-device systems, followed by a three-step post-warming dilution for 11 minutes. Comparative UFFT treatment used a strict 1-min equilibration solution (ES)/1-min vitrification solution (VS) (Gallardo, 2018; Gallardo et al., 2019) before rapid cooling directly in liquid nitrogen on open-system devices (two oocytes/device). Vitrification dilutions were performed in 50–100-µl droplets in 60mm embryo culture dishes. All oocytes experienced rapid warming in 2 mL of 37°C Kitazato thaw solution, with all UFFT-treated oocytes undergoing rapid elution of CPA before placement into 1 mL of warm (37°C) Global Total (High Protein, LGPS 10 mg/mL) for rehydration for an hour. After rehydration in GT-HP, all oocytes were cultured in 25-µL microdroplets of single-step Global Total (GT) (LGPS 5 mg/mL) culture medium under LifeGlobal light oil in tri-gas incubators (7.5% CO_2, 5% O_2).

Morphology Assessment

Using polarized light microscopy, post-thaw MII oocytes were examined for spindle integrity and polar body morphology. Spindle integrity was defined as continuous birefringence and its shape, and polar body morphology as intact, spherical, and non-fragmented. Additionally, oocytes were analyzed for degeneration after three hours of culture in GT medium.

PI⁺ EV Analysis

Oocytes were cultured for three hours after thawing in GT medium. EVs from culture supernatants were isolated via differential centrifugation followed by ultracentrifugation at 100,000 × g. PI staining (5 µg/mL, 15 min, RT) was followed by flow cytometry analysis.

mtUPR Gene Expression

Pools of 80 oocytes per replicate (n = 16) underwent RNA extraction. cDNA was synthesized, and qRT-PCR was performed to quantify Hsp60, Atf5, and ClpP, with Gapdh as the housekeeping gene. ΔΔCt was used for relative quantification.

Statistics

Data are mean ± SEM. One-way ANOVA with Tukey's post-hoc was used; $p < 0.05$ was considered significant.

Results

mtUPR Activation

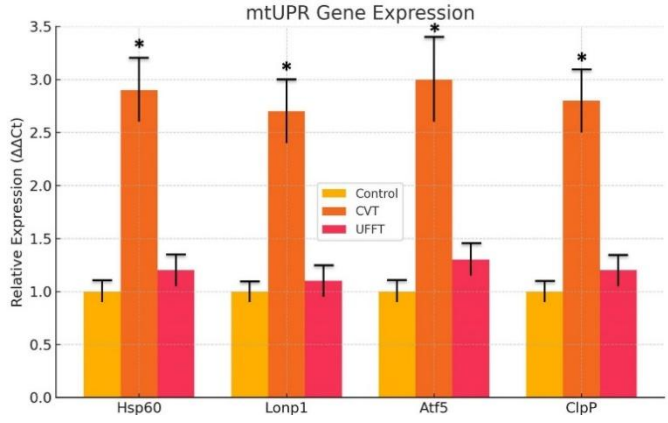
mtUPR gene expression was highest in CVT for all markers (Hsp60: 2.9 ± 0.3; Lonp1: 2.7 ± 0.3; Atf5: 3.0 ± 0.4; ClpP: 2.8 ± 0.3). UFFT values were intermediate and significantly lower than CVT ($p < .001$), but not significantly different from control ($p > .05$) (Figure 1).

PI⁺ EV Content

PI⁺ EV fraction was 34.0 ± 4.0% in CVT, significantly higher than UFFT (18.0 ± 3.0%, $p < .001$) and control (5.0 ± 1.0%, $p < .001$). Additionally, the PI⁺ EV fraction in the UFFT group was significantly higher than the control group ($p <. 05$) (Figure 2).

Figure 1.

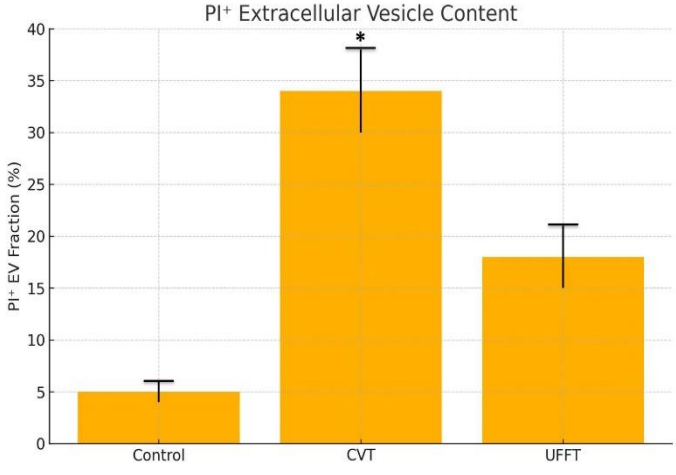
mtUPR gene expression in mouse oocytes after cryopreservation. Relative expression levels (ΔΔCt) of Hsp60, Lonp1, Atf5, and ClpP in control (fresh), CVT, and UFFT groups. CVT oocytes exhibited significantly higher expression than controls and UFFT for all genes ($p < .001$).



Note: Bars represent mean ± SEM (n = 16 replicates of 80 oocytes each). * indicates a significant difference between control and UFFT. Hsp60: chaperonin, Lonp1: lon peptidase 1, Atf5: activating transcription factor 5, ClpP: caseinolytic mitochondrial matrix peptidase proteolytic subunit.

Figure 2.

Proportion of PI⁺ EVs released post-thaw. PI⁺ EV fraction was significantly higher in CVT than in control and UFFT ($p < .001$).



Note: Bars represent mean ± SEM from three independent experiments. * indicates a significant difference between control and UFFT. CVT: Conventional vitrification, UFFT: Ultrafast freeze–thaw.

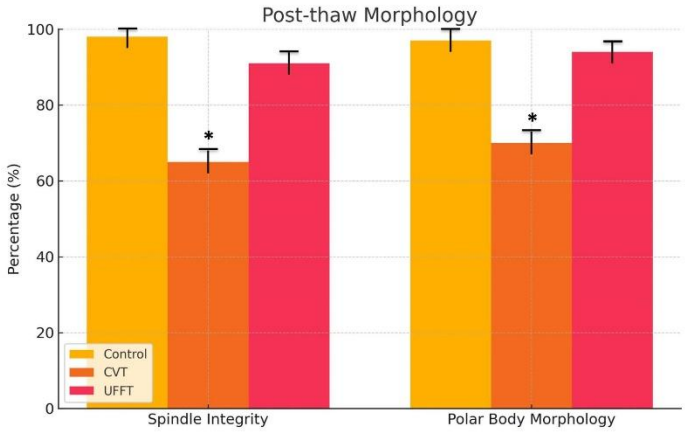
Morphology

Spindle integrity was preserved in 98% of control oocytes, 65% of CVT, and 91% of UFFT ($p< .001$ UFFT vs CVT). Polar

body morphology followed similar trends (97%, 70%, and 94% for control, CVT, and UFFT, respectively). There is no statistically significant difference between degeneration percentages after thaw (96.5% vs 95.5% UFFT vs CVT, $p > .05$) (Figure 3).

Figure 3.

Post-thaw spindle integrity and polar body morphology in mouse oocytes. CVT oocytes showed significantly reduced spindle and polar body preservation compared to control and UFFT (p< .001).



Note: Bars represent mean ± SEM from three independent experiments. * indicates a significant difference between control and UFFT.

Discussion

The present study provides a comparative evaluation of UFFT and CVT in a murine model, focusing on subcellular integrity and molecular stress responses. Our findings demonstrate that UFFT significantly mitigates cryopreservation-induced damage compared to the standard CVT protocol. Specifically, UFFT preserved meiotic spindle organization and polar body retention at levels comparable to those of fresh controls, reduced membrane necrosis, as evidenced by lower PI+ EV release, and maintained mitochondrial proteostasis by preventing the activation of the mtUPR.

The preservation of the meiotic spindle is a primary determinant of oocyte developmental competence. Our data show that CVT resulted in a significant loss of spindle birefringence (65% integrity) and polar body fragmentation, whereas UFFT preserved these structures in more than 90% of oocytes. These results align with recent studies suggesting that ultra-rapid cooling rates achieved by UFFT (>105 °C/min) enable the oocyte to bypass the critical temperature zone for tubulin depolymerization more effectively than conventional methods (Gallardo et al., 2019; Liebermann et al., 2024). Furthermore, the minimization of exposure to CPAs in the UFFT protocol—reduced to two minutes total—likely attenuates the osmotic shock that contributes to cytoskeletal disassembly, a protective effect recently corroborated in mouse oocytes where UFFT showed superior preservation of endoplasmic reticulum and mitochondrial distribution compared to CVT (Cho et al., 2024; Martinez-Rodero et al., 2025).

A novel aspect of this study was the evaluation of mitochondrial stress via the mtUPR pathway. Mitochondria are exquisitely sensitive to cryoinjury, and the accumulation of unfolded or oxidatively damaged proteins in the matrix triggers the upregulation of chaperones and proteases. We observed a robust activation of Hsp60, Lonp1, Atf5, and ClpP in the CVT group, indicating significant proteotoxic stress. In contrast, UFFT oocytes exhibited expression profiles indistinguishable from those of fresh controls. Recent transcriptomic analyses have highlighted Atf5 as a critical regulator of the oocyte's response to vitrification stress; its downregulation is associated with improved mitochondrial membrane potential and recovery (Zhou et al., 2024). Similarly, the protease ClpP is essential for maintaining oocyte competence; its deficiency leads to follicular depletion and subfertility (Wang et al., 2018; Yildirim et al., 2022). The absence of ClpP upregulation in UFFT oocytes suggests that the ultrafast protocol avoids the protein aggregation that necessitates this repair mechanism, potentially preserving the oocyte’s energetic reserves for fertilization.

Finally, we used flow cytometry to quantify PI+ EVs in the culture medium as a noninvasive biomarker of membrane necrosis. While EVs are standard mediators of intercellular communication, the presence of PI+ vesicles indicates membrane permeabilization and cell damage (Pallinger et al., 2017). The significantly elevated fraction of PI+ EVs in the CVT group (34%) reflects substantial membrane stress during the warming and rehydration phases. The reduction of this marker to 18% in the UFFT group confirms that the rapid elution and rehydration steps in the ultrafast protocol exert less mechanical shear stress on the plasma membrane. Although UFFT values remained higher than fresh controls, the significant improvement over CVT

supports the hypothesis that kinetic optimization can reduce sublethal membrane injury.

Conclusion

In conclusion, this study demonstrates that the UFFT protocol is superior to CVT for cryopreservation of mouse oocytes, with respect to cytoskeletal preservation, membrane integrity, and mitochondrial health. By minimizing CPA exposure and maximizing cooling/warming kinetics, UFFT prevents mtUPR activation and reduces necrotic EV release. These findings suggest that UFFT may offer a more physiological approach to gamete banking, potentially improving downstream developmental outcomes in assisted reproductive technologies.

Ethics Committee Approval: C57BL/6 female mice were maintained according to Yale University's requirements for animal research, and the Institutional Animal Care and Use Committee approved all procedures (Date: March 14 2022, protocol no. 2022-11300).
Author Contributions: MB is the sole author.
Peer-review: Externally peer-reviewed.
Funding: The author declared that this study has received no financial support.
Declaration of Interests: The author declares no conflicts of interest.
Use of Artificial Intelligence: All content, including text and data, is the original work of the author and was not generated by AI.

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