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Review | Derleme

MOLECULAR-MORPHOLOGICAL DISSOCIATION IN VITRIFIED-WARMED HUMAN EMBRYOS: RETHINKING EPIGENETIC RISK BEYOND MORPHOLOGY

VİTRİFİYE-ÇÖZÜLMÜŞ İNSAN EMBRİYOLARINDA MOLEKÜLER-MORFOLOJİK AYRIŞMA: EPİGENETİK RİSKİN MORFOLOJİNİN ÖTESİNDE YENİDEN DEĞERLENDİRİLMESİ

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ABSTRACT

Although vitrification has transformed assisted reproductive technology, it occurs during a critical developmental window characterized by extensive epigenetic remodeling and transcriptional recalibration; freeze-thaw procedures expose gametes and embryos to osmotic, cryoprotectant, oxidative, and mitochondrial stress, raising concerns about molecular effects beyond morphology. This structured narrative review, conducted without a predefined protocol, evaluates whether vitrification induces meaningful epigenetic disruption by integrating current human methylome, transcriptomic, and clinical outcome data. English-language literature was searched in PubMed/MEDLINE and Scopus, supplemented by manual screening of the reference lists of retrieved articles. Priority was given to human preimplantation embryo studies published up to January 2026, while mechanistic inferences were drawn selectively from oocyte, sperm, and animal-model data where direct human evidence was limited. Evidence derived from a limited number of primary human embryo studies generally suggests preservation of global DNA methylation architecture and overall transcriptional stability under contemporary protocols. Reported molecular alterations are predominantly localized and modest in magnitude, while available clinical data do not show a reproducible reduction in implantation or live birth rates associated with vitrification. The interaction between cryobiological stress and gamete-derived epigenetic architecture (paternal DNA methylation, chromatin organization, and small non-coding RNA cargo) may further influence embryonic sensitivity. Vitrification appears to be a biologically active yet developmentally buffered intervention; available data suggest that detectable molecular variation is generally limited rather than indicative of global epigenomic destabilization. Future stage-specific multi-omics studies integrated with longitudinal outcomes are required to define thresholds of biological relevance.

Keywords: Vitrification, Cryopreservation, DNA methylation, Gamete-derived epigenetics, Embryonic plasticity

ÖZ

Vitrifikasyon yardımcı üreme teknolojisini dönüştürmüş olsa da, kapsamlı epigenetik yeniden şekillenme ve transkripsiyonel yeniden kalibrasyonla karakterize kritik bir gelişim penceresinde gerçekleşir; dondurma-çözme işlemleri gamet ve embriyoları ozmotik, kriyoprotektan, oksidatif ve mitokondriyal strese maruz bırakarak morfolojinin ötesinde moleküler etki kaygıları doğurur. Önceden tanımlanmış bir protokole dayanmayan bu yapılandırılmış anlatı derlemesi, vitrifikasyonun anlamlı epigenetik bozulmaya yol açıp açmadığını mevcut insan metilom, transkriptomik ve klinik sonuç verileriyle değerlendirmeyi amaçlamaktadır. İngilizce literatür PubMed/MEDLINE ve Scopus'ta tarandı; alınan makalelerin kaynakçaları elle gözden geçirildi. Ocak 2026'ya kadar yayımlanan insan implantasyon öncesi embriyo çalışmalarına öncelik verildi; doğrudan insan kanıtının sınırlı olduğu durumlarda oosit, sperm ve hayvan modeli verilerinden seçici çıkarımlar yapıldı. Sınırlı sayıda birincil insan embriyosu çalışmasına dayanan kanıtlar, çağdaş protokoller altında global DNA metilasyon mimarisinin ve genel transkripsiyonel stabilitenin büyük ölçüde korunduğunu düşündürmektedir. Bildirilen moleküler değişiklikler çoğunlukla lokalize ve mütevazı büyüklüktedir; mevcut klinik veriler ise implantasyon veya canlı doğum oranlarında vitrifikasyonla ilişkili tekrarlanabilir bir azalma göstermemektedir. Kriyobiyolojik stres ile gamet kaynaklı epigenetik mimari (baba kökenli DNA metilasyonu, kromatin organizasyonu ve küçük kodlayıcı olmayan RNA yükü) arasındaki etkileşim de embriyonik duyarlılığı etkileyebilir. Vitrifikasyon biyolojik olarak aktif ancak gelişimsel olarak tamponlanmış bir müdahale gibi görünmektedir; mevcut veriler, saptanabilir moleküler varyasyonun genellikle sınırlı olduğunu ve global epigenomik istikrarsızlaşmayı göstermediğini düşündürmektedir. Biyolojik önem eşiklerini tanımlamak için uzunlamasına sonuçlarla entegre, aşamaya özgü çoklu-omik çalışmalar gereklidir.

Anahtar Kelimeler: Vitrifikasyon, Kriyoprezervasyon, DNA metilasyonu, Gamet kaynaklı epigenetik, Embriyonik plastisite

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Introduction

The widespread adoption of cryopreservation strategies has profoundly reshaped contemporary assisted reproductive technology (ART). Advances in vitrification protocols and the increasing implementation of elective freeze-all strategies have reinforced the perception that embryo cryopreservation is technically efficient and generally regarded as clinically safe.[1-7] However, preimplantation embryos undergo extensive epigenetic reprogramming during early development, characterized by genome-wide DNA demethylation, locus-specific remethylation, and tightly coordinated transcriptomic remodeling.[7-9] This period of dynamic epigenetic plasticity temporally overlaps with routine laboratory manipulations, including vitrification and warming. Experimental and mechanistic studies indicate that freeze-thaw procedures may induce osmotic stress, reactive oxygen species generation, mitochondrial perturbation, and ultrastructural alterations.[10-15] Although most embryos survive vitrification with preserved morphology, these biological stressors raise the question of whether subtle molecular adaptations may occur despite apparent structural recovery.

Available human embryo studies suggest broad preservation of global methylation and overall transcriptomic architecture after vitrification-warming, while also reporting localized epigenetic variation and pathway-specific transcriptional responses.[15-17] Experimental studies have additionally described regulatory RNA changes under specific conditions.[18-20] These findings suggest that morphological normality does not necessarily guarantee complete molecular equivalence. Taken together, current evidence supports a model of predominant molecular stability with potential for limited region-specific variability within the physiologically dynamic landscape of early embryonic development.[7,8,15-17]

Against this background, a structured narrative evaluation of the sublethal molecular effects of freeze-thaw procedures is warranted.

This review therefore integrates evidence on embryonic epigenetic vulnerability, cryobiological stress mechanisms, and human molecular datasets to propose a conceptual framework for interpreting potential molecular-morphological dissociation in vitrified-warmed embryos.

Relevant English-language literature was identified through searches of PubMed/MEDLINE and Scopus, supplemented by manual screening of the reference lists of retrieved articles. Priority was given to human preimplantation embryo studies published up to January 2026, while mechanistic inferences were drawn selectively from oocyte, sperm, and animal-model data where direct human evidence was limited. As a structured narrative rather than systematic review, no predefined protocol registration or quantitative synthesis was undertaken.

Epigenetic Reprogramming and Developmental Vulnerability in Early Human Embryos

Following fertilization, the human embryo undergoes genome-wide DNA demethylation followed by stage-specific de novo remethylation prior to implantation. These lineage- and stage-dependent transitions represent a tightly regulated developmental program essential for early embryogenesis.[7] Single-cell methylome analyses demonstrate that epigenetic reprogramming is heterogeneous across blastomeres. While certain genomic regions resist demethylation, others undergo active remodeling during cleavage stages, coinciding with embryonic genome activation and early lineage specification.[7,8] Multi-omics data further indicate that DNA methylation dynamics are closely coupled with transcriptional regulation during this critical window.[8] Laboratory manipulations, including vitrification and warming, overlap temporally with this sensitive epigenetic phase. Experimental data indicate that freeze-thaw procedures may induce osmotic stress, oxidative imbalance, mitochondrial perturbation, and cytoskeletal disruption.[11-15] This raises the possibility that molecular alterations may occur despite preserved morphology.

Global DNA demethylation and remethylation: Global DNA methylation levels decline rapidly after fertilization, reflecting active demethylation of the paternal genome and passive demethylation of the maternal genome across early cleavage stages.[7] This genome-wide erasure is followed by gradual, stage-specific remethylation prior to implantation. These processes are tightly regulated rather than stochastic. Single-cell methylome studies show that promoters, repetitive elements, and regulatory regions display differential susceptibility to methylation loss and regain, coordinated with embryonic genome activation and lineage specification.[7,8] Even modest perturbations during this window may influence downstream transcriptional programming.

Imprinting stability during reprogramming: Imprinted loci are relatively protected from global demethylation and preserve parent-of-origin-specific methylation marks essential for normal embryonic and placental development.[7] However, this protection is not absolute. Human embryo data indicate broadly preserved methylation of examined imprinted genes after vitrification-warming, although localized differentially methylated regions have been reported elsewhere in the genome.[16] In contrast, murine models have demonstrated imprint-related gene expression changes under certain cryopreservation conditions.[21] These findings suggest that imprinting is relatively robust but remains susceptible to environmental perturbation.

Transcriptome-epigenome coupling: Preimplantation development involves tightly coordinated DNA methylation remodeling and transcriptional activation. Multi-omics analyses demonstrate strong coupling between epigenetic states and gene expression during cleavage and blastocyst stages.[8] Human embryo studies

evaluating vitrification-warming have reported region-specific DNA methylation differences[16] and selected transcriptomic changes, including stress-responsive pathways.[15,17] Long-term storage analyses similarly indicate preservation of overall methylation and RNA expression profiles.[16,17] Overall, transcriptome-epigenome coupling appears robust yet sensitive to environmental influences. Whether observed molecular variability represents adaptive recalibration or biologically meaningful perturbation remains unresolved.[15-17]

Cryobiological Stress During Freeze-Thaw Procedures

Cryopreservation exposes gametes and embryos to rapid physicochemical stress characterized by abrupt osmotic, thermal, and metabolic shifts.[22,23] Although vitrification prevents intracellular ice crystal formation, it requires high cryoprotectant concentrations and rapid osmotic transitions during cooling and warming, generating cellular stress beyond morphological detectability. Oxidative stress represents a central mechanism of cryoinjury. Increased reactive oxygen species (ROS) production has been demonstrated during cryopreservation, contributing to membrane destabilization, mitochondrial dysfunction, and DNA damage.[11,12,24] Mitochondria constitute a key vulnerability node, with experimental data showing alterations in ultrastructure and membrane potential after vitrification.[12,14] Given the metabolic demands of preimplantation development, even moderate mitochondrial dysfunction may influence downstream cellular processes.[7,8]

Ultrastructural analyses of vitrified human embryos demonstrate preserved gross morphology alongside detectable subcellular alterations, indicating that conventional light microscopy may underestimate cellular stress.[14] Intervention studies using antioxidant supplementation or mitochondrial stabilization further support the biological relevance of cryogenic stress.[25,26]

Taken together, freeze-thaw procedures introduce oxidative, osmotic, metabolic, and structural stressors that provide a mechanistic basis for sublethal molecular perturbation.[11-15,22,23]

Osmotic and thermal stress: Vitrification involves rapid exposure to concentrated cryoprotectant solutions followed by ultra-rapid cooling and warming, generating abrupt osmotic gradients across cell membranes.[2,25] Water flux during equilibration and dilution phases induces transient cellular shrinkage and re-expansion, creating mechanical strain on membranes and cytoskeletal structures. Thermal fluctuation alters membrane fluidity, protein conformation, and mitochondrial stability, while rapid cooling-rewarming may disrupt cytoskeletal organization despite acceptable post-thaw survival rates.[1,6] Ultrastructural assessments confirm that preserved morphology does not necessarily indicate subcellular integrity.[14]

ROS and mitochondrial perturbation: ROS generation is a recognized consequence of cryopreservation, promoting mitochondrial electron leakage and metabolic imbalance, demonstrated predominantly in gamete and animal models.[11,12,24] Given the close link between mitochondrial metabolism and epigenetic regulation—via redox-dependent modulation of DNA methyltransferases and histone-modifying enzymes—oxidative stress may intersect with ongoing epigenetic remodeling during early development.[7,8] Experimental studies demonstrate that antioxidant and metabolic support strategies partially restore post-thaw developmental competence, supporting the mechanistic role of ROS-mediated perturbation.[22,26] Protective cryopreservation approaches have likewise been investigated across diverse reproductive cell and tissue types.[27-29]

Cryoprotectant exposure and ultrastructural changes: Cryoprotectants such as dimethyl sulfoxide (DMSO) and ethylene glycol impose concentration- and exposure-dependent chemical stress.[25] Cryopreservation protocols continue to be optimized for limited and specialized reproductive tissues.[30] Ultrastructural studies of vitrified embryos show preserved morphology alongside subtle mitochondrial and cytoplasmic alterations.[14] Although many embryos recover after warming, these findings confirm that cryoprotectant exposure is biologically active rather than inert.[1,2,6,25] Current human molecular datasets do not demonstrate systematic epigenomic destabilization following vitrification; however, the biologically active nature of cryoprotectants and osmotic stress supports the need for high-resolution molecular investigation.[15-17,23]

Molecular and Epigenetic Evidence After Vitrification

While cryobiological stress provides a mechanistic basis for subcellular and metabolic perturbation, the key question is whether such stress results in measurable molecular or epigenetic alterations in human embryos. Human studies have moved beyond morphology to examine DNA methylation, transcriptomic profiles, and mitochondrial responses.[15-17] Current data do not support widespread epigenomic destabilization following vitrification.[15-17] However, region-specific methylation differences and selected transcriptomic changes have been reported.[15-17] Long-term storage studies similarly indicate preservation of global methylation and mRNA/long non-coding RNA (lncRNA) expression profiles.[16,17] Collectively, these findings suggest that vitrification does not induce large-scale epigenetic disruption, although subtle molecular deviations cannot be excluded. Within the dynamic framework of preimplantation epigenetic remodeling, such changes may reflect adaptive recalibration rather than pathological destabilization.[7,8,15-17] Importantly, these conclusions derive from a limited number of primary human embryo datasets and should therefore be regarded as provisional.

DNA methylation landscape after vitrification: Genome-wide analyses indicate broadly similar global methylation patterns in fresh and vitrified-warmed human embryos.[16] Nevertheless, localized differentially methylated regions have been reported.[16] These alterations are region-

specific, and no significant differences were observed in the methylation levels of examined imprinted genes.[16] Studies evaluating prolonged cryostorage indicate that storage duration does not substantially alter global DNA methylation patterns, supporting the stability of the embryonic methylome under contemporary protocols.[16] Overall, available data do not indicate large-scale disruption of DNA methylation but remain compatible with subtle, locus-specific variation of uncertain biological significance.[16]

Transcriptomic alterations and regulatory RNAs: Human transcriptomic studies suggest that overall expression architecture is broadly preserved, while selected mRNA/lncRNA and pathway-level changes can occur after vitrification-warming.[15,17] Reported changes include stress-response, metabolic, apoptotic, cell-cycle, cell-adhesion, and developmental signaling pathways.[15,17] Experimental data suggest that regulatory RNAs, including microRNAs, may be sensitive to cryobiological stress, although direct human evidence remains limited.[18] In addition, sperm-derived small non-coding RNAs contribute to early embryonic regulation, but the impact of cryopreservation on these pathways is not well defined.[31]

Taken together, vitrification-warming does not appear to cause global transcriptional dysregulation, but selected transcriptomic changes have been documented.[15,17]

Evidence from imprinting and locus-specific analyses: Imprinted regions are relatively protected during epigenetic reprogramming. Available human embryo methylome data did not show significant differences in the methylation levels of examined imprinted genes after vitrification-warming, despite the presence of region-specific differentially methylated regions elsewhere in the genome.[16] Current human data therefore do not support systematic imprinting instability under contemporary vitrification protocols.[1,4,6,16] Although murine models suggest potential imprint-related alterations under certain conditions,[21] the human evidence remains limited.

Stability versus adaptive response: A key question is whether observed molecular variations represent sublethal injury or adaptive responses. Limited human studies suggest broad preservation of global methylation and overall transcriptomic architecture following vitrification-warming, while localized or pathway-specific changes have also been reported.[15-17] Given the inherently dynamic and plastic nature of early embryonic development, such variations may fall within physiological limits, but their biological significance remains uncertain. At the same time, the sensitivity of regulatory networks raises the possibility that subtle alterations could have downstream effects, although available clinical outcome data do not indicate a consistent reduction in implantation or live birth following vitrification.[32,33] Overall, the evidence supports a model of relative molecular stability with limited region-specific variability. Whether these changes represent stochastic variation, adaptive recalibration, or rare sublethal perturbation remains unresolved. High-resolution, stage-matched studies integrating molecular and clinical outcomes are required to clarify this distinction.[7,8,15-17] Until such evidence emerges, vitrification may be regarded as a biologically active yet generally developmentally buffered process, without current evidence of systematic epigenomic destabilization.[15-17]

Laboratory Modifiers and Technical Determinants

Cryobiological outcomes are influenced not only by vitrification itself but also by laboratory-specific variables, including protocol design, cryoprotectant formulation, system configuration, and repeated embryonic manipulations.[1,6,14,23] These factors affect cooling kinetics, osmotic gradients, and cryoprotectant exposure, thereby modulating oxidative and metabolic stress during freeze-thaw procedures.[14,22,25,26] Such variability may partly explain differences observed across experimental and clinical studies, particularly when subtle molecular endpoints are assessed.[6,15-17,23] Interpretation of vitrification-associated findings therefore requires consideration of laboratory context rather than freezing status alone.

Closed versus open vitrification systems: Open systems allow direct contact with liquid nitrogen and enable ultra-rapid cooling, whereas closed systems reduce contamination risk but may alter thermal exchange dynamics.[6,14,23] Ultrastructural analyses of human embryos demonstrate preserved morphology across open and closed systems, although subcellular alterations may be detected with high-resolution imaging.[14] Direct molecular comparisons between open and closed embryo vitrification systems remain limited. Human oocyte transcriptomic data from closed-system approaches suggest that system-specific molecular effects require further study.[19] Thus, potential differences in cooling kinetics and osmotic exposure should be considered without assuming molecular equivalence between system types.

Cryoprotectant composition: Cryoprotectant formulation is a key determinant of cellular response during vitrification. Agents such as DMSO and ethylene glycol induce osmotic and chemical stress that may affect membrane integrity and intracellular signaling.[22,23] Experimental modifications, including the addition of late embryogenesis abundant (LEA) proteins, fatty acids, L-carnitine, and antioxidant compounds, have been reported to improve survival, mitochondrial function, or developmental competence in oocyte and animal models.[20,22,26] These findings support the concept that cryoprotectant exposure is biologically active rather than inert. Although comparative human data on methylation profiles across formulations are limited, current evidence suggests broad epigenetic stability under contemporary protocols.[15-17] Whether formulation-dependent stress differences result in stable epigenetic changes or transient adaptation remains unclear.

Mitochondrial and oxidative modulation strategies: Mitochondria represent a central vulnerability during cryopreservation due to their roles in ATP production and

redox regulation. Freeze-thaw procedures may disrupt mitochondrial membrane potential, alter ultrastructure, and increase ROS generation.[11,12,22,26] Given the close link between mitochondrial metabolism and epigenetic regulation, oxidative imbalance may influence DNA methyltransferase activity and chromatin-modifying enzymes during early development.[7,8] Studies in gamete and animal models suggest that antioxidant and metabolic support strategies may improve mitochondrial integrity and developmental competence following cryopreservation, supporting the relevance of ROS-mediated mechanisms.[11,12,22,24,26,34] Overall, oxidative stress and mitochondrial instability are key mediators of cryobiological stress, although current human data do not indicate widespread epigenetic destabilization.[15-17]

Repeated manipulation effects: In modern ART practice, embryos may undergo multiple interventions, including trophectoderm biopsy and repeated vitrification-warming cycles, particularly in preimplantation genetic testing (PGT) programs.[35,36] Meta-analyses suggest that repeated biopsy and/or vitrification may be associated with lower clinical pregnancy and live birth/ongoing pregnancy rates compared with single biopsy and single vitrification, although the underlying studies are predominantly retrospective and neonatal data remain limited.[35,36] Available molecular datasets primarily reflect single vitrification events, and the molecular effects of cumulative exposure remain insufficiently characterized.[15-17] Thus, repeated embryonic manipulation warrants cautious clinical interpretation and further molecular investigation.

Gamete-Derived Epigenetic Background and Cryo Interaction

Cryopreservation-related molecular effects should not be considered at the embryo level alone, as early development is shaped by epigenetic information inherited from both gametes.[7,8,31] Following fertilization, the zygote undergoes extensive genome-wide epigenetic reprogramming, during which paternal and maternal epigenetic marks are differentially remodeled.[7,8] Within this dynamic window, the composition and integrity of gamete-derived epigenetic components may influence early developmental regulation. Paternal contributions extend beyond DNA sequence to include locus-specific DNA methylation patterns, retained histones, and small non-coding RNA (sncRNA) cargo involved in early gene regulation.[7,8,31] These elements are introduced during rapid chromatin reorganization, suggesting that cryopreservation-induced perturbations at the gamete level may intersect with embryonic epigenetic plasticity.

Sperm sncRNA contribution: Spermatozoa deliver diverse sncRNAs, including microRNAs, PIWI-interacting RNAs (piRNAs), and tRNA-derived fragments, which contribute to post-transcriptional regulation and early developmental programming.[7,8,31] Variations in sncRNA composition may influence early regulatory network stability. Although direct human evidence remains limited, RNA-mediated paternal inheritance provides a framework through which cryopreservation-related alterations could affect embryonic development. Integrated studies combining sperm cryostorage status with embryonic transcriptomic and methylomic profiling remain scarce.

DNA fragmentation and oxidative load: Sperm cryopreservation is associated with increased ROS production and elevated DNA fragmentation.[11,24] Oxidative stress contributes to membrane instability, mitochondrial dysfunction, and genomic damage. During early embryogenesis, the paternal genome undergoes rapid active demethylation.[7,8] In this context, pre-existing DNA fragmentation or oxidative lesions may influence chromatin remodeling and epigenetic resetting. Although techniques such as intracytoplasmic sperm injection (ICSI) enable fertilization, they do not eliminate underlying molecular damage.[24] The interaction between paternal oxidative load and embryonic epigenetic plasticity remains insufficiently defined, and high-resolution human studies integrating sperm DNA integrity with embryonic molecular profiling are limited.

Paternal cryopreservation effects: Sperm cryopreservation may also alter chromatin organization and small RNA composition.[11,24,31] While clinical data do not demonstrate consistent adverse outcomes, subtle molecular effects cannot be excluded. Because the embryo inherits both genetic and epigenetic information from gametes, cumulative stress exposure—particularly when both gametes and embryos are cryopreserved—may influence developmental trajectories. However, integrated human studies addressing this interaction remain limited.

Overall, gamete-derived epigenetic background represents a potential modifier of cryobiological response. Its contribution to sublethal molecular variation in human embryos remains an important area for further investigation.

Repeated Manipulation: PGT, Double Biopsy and Double Vitrification

Current meta-analyses suggest that repeated blastocyst biopsy and/or vitrification may be associated with reduced clinical pregnancy and live birth/ongoing pregnancy rates compared with single biopsy and vitrification in euploid embryo transfer cycles.[35,36] The certainty of the evidence is limited because the available studies are observational, and neonatal outcomes are sparsely reported.[35,36] Corresponding molecular evidence remains limited, as most human methylome and transcriptomic studies evaluate single vitrification events.[15-17] Direct assessment of cumulative freeze-thaw exposure at the level of locus-specific methylation, chromatin architecture, or regulatory RNA networks is scarce. Within the dynamic framework of preimplantation epigenetic remodeling—characterized by genome-wide demethylation, lineage-specific remethylation, and coordinated transcriptional recalibration—subtle perturbations may remain undetectable using bulk analytical approaches. The presence of clinical associations does not establish the underlying molecular mechanism, and cumulative

molecular effects remain unresolved. High-resolution studies integrating cumulative procedural exposure with single-cell epigenomic and transcriptomic profiling, combined with longitudinal clinical follow-up, are required to clarify these relationships.[7,8,15-17]

Clinical Correlates: When Does It Become Meaningful?

The clinical relevance of molecular or epigenetic alterations associated with cryopreservation depends on whether these changes translate into measurable outcomes such as implantation, live birth, perinatal results, or long-term offspring health.[32,33] Although subcellular stress and localized molecular variation may be detectable,[15-17] clinical significance requires consistent impact on reproductive endpoints. Available human data suggest broadly preserved global DNA methylation patterns and overall transcriptomic architecture, despite localized or pathway-specific differences.[15-17] Consistently, large-scale analyses comparing fresh and frozen embryo transfer (FET) cycles show no reproducible reduction in implantation or live birth rates associated with cryopreservation.[32,33]

Obstetric outcomes after FET remain heterogeneous and are influenced by multiple factors, including patient characteristics, embryo stage, biopsy status, freeze-all strategies, and endometrial preparation protocols.[32,33,37,38] Interpretation of these findings requires caution. The absence of reduced reproductive outcomes does not exclude subtle molecular variation,[15-17] while vascular or hypertensive phenotypes observed after FET are more likely related to endometrial and hormonal factors than to direct embryo-derived epigenetic disruption.[37,38]

Within the context of early embryonic development—characterized by extensive epigenetic remodeling—minor molecular deviations may fall within physiological plasticity rather than representing pathological injury.[7,8] The threshold at which such variation becomes clinically meaningful remains unclear.

Implantation and live birth rates: Meta-analyses comparing fresh and frozen embryo transfer cycles demonstrate comparable implantation and live birth rates, with some studies suggesting improved outcomes in selected populations following frozen transfer strategies.[32,33] Similarly, freeze-all approaches in conditions such as adenomyosis and endometriosis do not show consistent disadvantage compared with fresh transfer.[39] Overall, current evidence does not indicate a reproducible reduction in reproductive success associated with vitrification.[32,33]

Obstetric and vascular phenotype: While reproductive outcomes are preserved, certain studies report an increased risk of hypertensive disorders, including preeclampsia, in specific FET protocols.[37,38,40] These associations are strongly influenced by endometrial preparation. Artificial cycle FET, characterized by the absence of a corpus luteum, is associated with higher rates of hypertensive and placental complications compared with natural or ovulatory cycles.[38] These findings suggest that endocrine and endometrial factors play a central role in shaping obstetric outcomes.

Artificial versus natural FET cycles: Differences between artificial and natural FET cycles are critical for interpreting obstetric data. Increased vascular risk in artificial cycles has been linked to altered endocrine signaling and absence of corpus luteum-derived factors.[37,38] This supports the interpretation that observed obstetric differences are more closely related to endometrial environment than to cryopreservation itself.

Interpreting inconsistency in clinical data: Heterogeneity in clinical findings reflects variation in study design, patient populations, embryo characteristics, and treatment protocols.[32,33,37,38] Available molecular data suggest broad global stability rather than complete locus-specific equivalence.[15-17] Overall, vitrification does not appear to reduce reproductive success, while observed obstetric differences are more consistently explained by maternal and endometrial factors than by embryo-derived molecular injury.[32,33,37,38] The clinical significance of sublethal molecular variation therefore remains context-dependent and incompletely defined.

Molecular-Morphological Dissociation: A Conceptual Synthesis

Conventional embryo assessment in ART remains predominantly morphology-based, relying on post-warming survival, blastocoel re-expansion, cell number, symmetry, and trophectoderm grading. In contrast, preimplantation development involves extensive epigenetic remodeling and coordinated transcriptional transitions,[7,8] raising the question of whether morphologically normal embryos may harbor subtle molecular variation.

Available human methylome and transcriptomic analyses suggest broad preservation of global molecular architecture after vitrification-warming, while localized methylation differences and selected gene-expression changes have also been reported.[15-17] These observations support the concept of molecular-morphological dissociation, in which structural integrity and developmental competence may coexist with minor molecular variability. Given the inherently plastic nature of early embryogenesis, such variation may fall within physiological limits rather than representing pathological injury.[7,8] Clinical data further support a generally reassuring interpretation. Meta-analyses indicate no consistent reduction in implantation or live birth rates following vitrification.[32,33] Reported obstetric differences, particularly hypertensive or vascular phenotypes, are more strongly associated with endometrial preparation protocols than with cryopreservation itself.[37,38] Importantly, the absence of adverse clinical outcomes in broader FET literature does not exclude subtle molecular variation,[15-17] while the presence of locus-specific epigenetic differences does not necessarily imply developmental harm. These findings raise the possibility that observed molecular changes reflect stochastic

variability or adaptive responses rather than sublethal injury.

Overall, current evidence supports a model of molecular resilience with limited region-specific variability. Molecular-morphological dissociation should therefore be interpreted as a framework distinguishing detectable molecular sensitivity from clinically meaningful impairment. Further clarification will require stage-specific single-cell multi-omics studies integrated with longitudinal clinical outcomes.[7,8,15-17] Emerging noninvasive approaches, such as analysis of embryo culture media secretome, may provide additional insight beyond conventional morphology-based assessment.[23]

Proposed Sublethal Epigenetic Signature Framework

Current human evidence does not support widespread epigenomic destabilization following vitrification-warming, although localized methylation and transcriptomic changes have been reported.[15-17] Together with evidence of mitochondrial and oxidative perturbation during cryopreservation,[11,12,14,24,26] these findings suggest the need for an integrative framework linking molecular sensitivity with preserved clinical outcomes.

The proposed framework conceptualizes vitrification not as a trigger of global epigenetic disruption, but as a source of localized molecular variation, subtle transcriptomic modulation, and transient metabolic-epigenetic recalibration. These changes are proposed to occur within the physiological plasticity of early embryonic development, characterized by dynamic epigenetic remodeling and transcriptional adaptation.[7,8] Within this framework, vitrification-associated molecular deviations are hypothesized to remain below the threshold required to impair implantation or live birth.[32,33] Molecular perturbations are therefore hypothesized to be subclinical and buffered by intrinsic embryonic regulatory resilience. Importantly, this framework describes a spectrum ranging from complete molecular stability to rare instances of biologically meaningful perturbation, with an intermediate zone of adaptive recalibration lacking measurable clinical consequences. This interpretation aligns with clinical data demonstrating preserved reproductive outcomes following vitrification,[32,33] while recognizing that certain obstetric phenotypes are more closely related to endometrial preparation than to cryopreservation itself.[37,38] This framework distinguishes detectable molecular sensitivity from clinically significant impairment, without implying concealed developmental injury. Validation of this framework will require stage-specific single-cell multi-omics analyses integrated with longitudinal clinical outcomes.[7,8,15-17] Current evidence remains limited by reliance on single vitrification events and bulk analytical approaches,[15-17] and cumulative procedural exposure remains insufficiently characterized at the molecular level.[35,36] Longitudinal integration of embryo-level molecular data with clinical outcomes is also lacking, and future studies should address implantation, placental function, and long-term offspring health.[32,33,37,38] In addition, the interaction between gamete-derived epigenetic background and embryo cryopreservation warrants further investigation.[24,31] Standardized methodologies and adequately powered studies will be required to define thresholds of biological relevance. Overall, integrative and longitudinal approaches are needed to determine whether vitrification-associated molecular variation represents adaptive plasticity or clinically meaningful alteration.

The framework yields several testable predictions. First, vitrification-associated differentially methylated regions are expected to localize predominantly outside canonical imprinting control regions and to remain modest in effect size. Second, candidate molecular readouts—locus-specific methylation at stress-responsive and metabolic loci, sperm-derived small non-coding RNA (microRNA and tRNA-derived fragment) profiles, and mitochondrial membrane potential or mitochondrial DNA (mtDNA) copy number—are predicted to show only minor, non-progressive shifts. Third, these molecular deviations are expected not to correlate with implantation or live birth rates. Each prediction is empirically testable using stage-matched single-cell multi-omics integrated with longitudinal clinical outcomes.

A structured summary of cryobiological stress pathways, molecular findings, and clinical correlates is presented (Table 1).

Conclusion

Cryopreservation exposes gametes and embryos to osmotic shifts, cryoprotectant exposure, and rapid thermal fluctuation. Although vitrification prevents intracellular ice formation, it remains a biologically active process. Vitrification-associated molecular changes appear predominantly localized rather than systemic and are not consistently associated with impaired blastocyst development or reduced implantation potential. Thus, vitrification acts on a developmentally heterogeneous substrate, and its molecular impact may be modulated by inherited epigenetic variability. The interaction between cryobiological stress and gamete-derived chromatin states may define thresholds of molecular sensitivity, consistent with a model in which cryobiological stress operates within the limits of intrinsic embryonic plasticity. Future stage-matched, single-cell multi-omics studies integrated with longitudinal reproductive and perinatal outcomes will be required to define thresholds of biological relevance and to further delineate the relationship between molecular sensitivity and developmental stability.

Limitations

This article is a structured narrative review rather than a systematic review; it did not follow a predefined search protocol or formal inclusion and exclusion criteria and is therefore subject to selection bias. Human embryo-specific molecular datasets remain limited and heterogeneous, with most relying on single vitrification events and bulk analytical approaches. Several

mechanistic inferences draw on oocyte, sperm, and animal-model data, which may not fully translate to the human embryo. Finally, the proposed conceptual framework has not been experimentally validated and requires confirmation in stage-specific, multi-omics studies integrated with longitudinal clinical outcomes.

Ethics Statement: This study is a review of previously published literature and does not involve human participants or animal experiments. Ethical approval was not required.

Table 1. Cryobiological stress pathways, molecular findings, and clinical correlates in human embryos.

Domain	Mechanistic Basis	Human Molecular Findings	Clinical Evidence	Integrated Interpretation
Mitochondrial Stress	ROS generation and mitochondrial membrane potential alterations reported in gamete and animal models[11,12,24,26]	mtDNA and stress-responsive transcriptomic changes reported in human blastocysts[15]; available methylation data do not indicate global epigenomic destabilization[16]	No consistent reduction in implantation or live birth in large analyses[32,33]	Metabolic stress detectable; developmental consequences remain uncertain
DNA Methylation	Stress during active demethylation/remethylation window[7,8]	Broadly similar genome-wide methylation with region-specific DMRs reported[16]	No reproducible adverse reproductive outcomes in human studies[32,33]	Localized variability of uncertain biological significance
Transcriptomic Regulation	Stress-responsive gene modulation	Overall transcriptomic architecture broadly preserved, with selected mRNA/lncRNA and pathway-level changes[15,17]	Direct clinical linkage between the reported transcriptomic changes and reproductive outcomes remains insufficiently established[15,17]	Limited transcriptomic change without evidence of global regulatory disruption
Imprinting Stability	Protected but sensitive DMR maintenance phase[7,8]	No significant differences in methylation of examined imprinted genes in available human embryo methylome data[16]	No consistent clinical signal of imprinting-disorder phenotypes associated with vitrification[4]	Examined imprinting marks largely preserved
Gamete-Derived Epigenetic Background	Sperm sncRNA cargo, oxidative DNA damage[24,31]	Limited embryo-integrated human datasets	No direct clinical linkage established in human studies	Potential modulator of molecular sensitivity thresholds
Repeated Manipulation (PGT, double vitrification)	Cumulative osmotic/oxidative exposure during repeated biopsy and vitrification[35,36]	Limited embryo-level molecular data on cumulative biopsy and vitrification exposure	Meta-analyses suggest lower clinical pregnancy and live birth/ongoing pregnancy rates after repeated biopsy and/or vitrification; evidence is largely retrospective[35,36]	Potential adverse clinical association with an underexplored molecular dimension
Obstetric Phenotypes after FET	Endometrial preparation-dependent vascular modulation	Direct embryo-level molecular links to later obstetric phenotypes remain insufficiently established	Increased risk of hypertensive disorders in artificial cycles[37,38]	Likely maternal/endometrial contribution rather than embryo-derived epigenetic disruption

ROS: reactive oxygen species; mtDNA: mitochondrial DNA; DMR: differentially methylated region; mRNA: messenger RNA; lncRNA: long non-coding RNA; sncRNA: small non-coding RNA; PGT: preimplantation genetic testing; FET: frozen embryo transfer.

Conflict of Interest
The authors declare that there is no conflict of interest regarding this study.

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Author Contributions

PCF; Conceptualization, study design, literature review, literature synthesis, and manuscript drafting; SF; Supervision, literature interpretation, critical revision, and final approval.

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