



## Review Article

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## Innovative strategies in sperm cryopreservation: Overcoming challenges and enhancing fertility preservation—A narrative review

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## ABSTRACT

Sperm cryopreservation is an essential technique for male fertility preservation, especially in men who are undergoing medical treatment. Conventional cryopreservation methods face limitations such as oxidative stress, DNA fragmentation, and cytotoxicity associated with traditional cryoprotectants like dimethyl sulfoxide (DMSO). Recent breakthroughs have focused on improving post-thaw sperm viability with novel cryoprotectants and innovative freezing strategies. Prospective approaches include the use of amino acid-based cryoprotectants, deep eutectic solvents, and antioxidants that have been described to prevent oxidative damage and maintain DNA integrity. Vitrification, a high-speed freezing technique that prevents ice crystal formation, has demonstrated superior outcomes compared to conventional slow freezing. Moreover, the Direct Dropping Method, a cryoprotectant-free approach, has been introduced as a contamination-minimizing technique that preserves sperm functionality. Multiomics tools are also utilized to determine biomarkers for protocol optimization. Despite these advancements, cryoprotectant toxicity is a central challenge, emphasizing the necessity for safer agents. Future research must focus on long-term sperm functionality and individualized cryopreservation strategies to maximize reproductive outcomes. The current review highlights the challenges associated with sperm cryopreservation, explores innovative strategies and novel cryoprotectants, underscores the significance of maintaining DNA integrity, and proposes future research directions to improve fertility preservation outcomes.

**KEYWORDS:** Assisted reproductive technology; Intracytoplasmic sperm injection; Cryoprotectants; Sperm cryopreservation; Dimethyl sulfoxide

## 1. Introduction

Cryopreservation enables long-term tissue storage by slowing metabolic processes at low temperatures. However, the formation of ice crystals during freezing can damage cellular integrity and disrupt the tissue's chemical composition[1]. Sperm cryopreservation is essential for male patients undergoing cancer treatment, as it enables the preservation of reproductive potential before the commencement of chemotherapy or radiotherapy[2]. For men with nonobstructive azoospermia, microdissection testicular sperm extraction (micro-TESE) is often necessary for sperm retrieval, making cryopreservation vital to avoid the need for multiple surgical procedures[3]. Various sperm cryopreservation techniques, including slow cooling and vitrification, commonly utilize glycerol and egg yolk buffers. Post-cryopreservation survival rates typically range from 40% to 60%. Additionally, emerging methods allow for the cryopreservation of individual sperm cells, which is especially beneficial in cases of limited sperm availability[4]. While cryopreservation preserves sperm viability, it can compromise DNA integrity, which is essential for successful fertilization and embryo development. As a result, innovative technologies are

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being developed to assess and reduce DNA damage[2]. The efficient management of cryo-storage facilities is essential for ensuring the safety and reliability of preserved specimens, highlighting the need for improved protocols[5].

Semen cryopreservation performed before undergoing antineoplastic treatment is the most effective approach for preserving male fertility, even in cases of abnormal semen parameters. The use of intracytoplasmic sperm injection (ICSI) allows for successful fertilization with a single sperm cell[6]. Recent advancements in oncology have improved survival rates among prepubertal boys, making cryopreservation of immature testicular tissue crucial for future fertility. Additionally, the shift from slow freezing to vitrification has improved sperm viability and assisted reproductive technology (ART) outcomes, with cryoprotectant selection and exposure protocols playing a critical role in sperm survival[7]. Diet, stress, and environmental factors contribute to reduced sperm quality, highlighting the importance of cryopreservation as a preventive measure for men at risk of fertility issues[8]. Sperm cryopreservation before medical transition offers transgender individuals the chance to preserve fertility, safeguarding future reproductive possibilities. This approach ensures that reproductive options remain available after transitioning[9].

The development of optimized, less toxic cryoprotectants is becoming increasingly important in cryopreservation, due to the limitations of traditional agents like dimethyl sulfoxide (DMSO). Conventional cryoprotectants often exhibit cytotoxicity, reduce cell viability, and pose challenges in clinical applications[10]. DMSO is widely used, but it has been shown to cause cell cycle arrest, apoptosis, and alterations in gene expression. These effects can negatively impact cellular integrity during cryopreservation[11].

The cytotoxic effects of DMSO necessitate post-thaw washing, which complicates its use in clinical settings. This additional step poses practical challenges in optimizing its application for cryopreservation[12]. Amino acids like *L*-proline and *L*-carnitine have shown effectiveness similar to DMSO, while exhibiting reduced cytotoxicity. These properties make them promising alternatives for cryopreservation[13]. *L*-methionine sulfoxide [Met(O)-OH] has shown significant ice recrystallization inhibition and reduced osmotic shock damage, effectively maintaining cell viability even at higher concentrations. This makes it a valuable agent for cryopreservation[11]. Emerging classes of cryoprotectants, including ionic liquids and deep eutectic solvents, are being explored for their lower toxicity profiles. These alternatives offer potential advantages in reducing cellular damage during cryopreservation[14]. Improved cryopreservation strategies are essential for the scalability of cell therapies, particularly in applications that require immediate post-thaw administration. These advancements are vital for ensuring the efficacy and viability of cells in therapeutic settings[12]. The aim of the review is to demonstrate the current challenges and advancements in sperm cryopreservation, focusing on optimizing post-thaw viability and DNA integrity. It highlights innovative

cryoprotectants and emerging techniques to improve fertility preservation outcomes.

## 2. Methodology

This narrative review examines recent advancements in sperm cryopreservation, emphasizing innovative strategies aimed at improving post-thaw viability and minimizing cytotoxic effects. To ensure the relevance and research integrity of the selected studies, inclusion criteria were established to encompass peer-reviewed articles published in high-impact journals specializing in reproductive medicine, cryobiology, and assisted reproductive technologies (ART) in the five-year span from 2019 to 2024. The review focused on key topics such as slow freezing, vitrification, and emerging cryoprotectant-free approaches. Studies assessing the effects of both conventional cryoprotectants (*e.g.*, glycerol, DMSO) and novel alternatives (*e.g.*, amino acids, ionic liquids) on post-thaw sperm viability, motility, and DNA integrity were included. Additionally, experimental, clinical, and *in vitro* research contributing to the understanding of cryoinjury mechanisms were considered. Conversely, studies published in languages other than English, research focusing on oocytes, embryos, or somatic cells, and articles lacking research integrity such as case reports, editorials, and non-peer-reviewed sources were excluded. Further exclusion criteria encompassed studies without quantitative evaluations of cryopreservation outcomes and research employing outdated techniques irrelevant to current clinical and laboratory practices. A comprehensive literature search was conducted using databases such as PubMed, Scopus, and Google Scholar, employing keywords including "sperm cryopreservation," "cryoprotectants," "vitrification," "oxidative stress," and "DNA integrity." Titles and abstracts were screened systematically, followed by an in-depth review of full-text articles. The extracted data were categorized based on cryopreservation methodologies, cryoprotectant efficacy, and post-thaw sperm quality parameters. Through the application of precise inclusion and exclusion criteria, this review provides a structured synthesis of contemporary advancements in sperm cryopreservation, with a particular focus on innovative techniques and their implications for fertility preservation. A total of 59 articles were selected and thoroughly reviewed, encompassing both literature analyses and detailed studies relevant to advancements in sperm cryopreservation, with an emphasis on enhancing viability and reducing toxicity.

## 3. Results

### 3.1. Cryoprotectants in sperm cryopreservation

Cryoprotectants are categorized into permeating and non-

permeating types based on their capacity to traverse cell membranes. Permeating cryoprotectants, such as glycerol and DMSO, penetrate the cell and provide intracellular protection against cryoinjury. In contrast, non-permeating agents, including sucrose and trehalose, remain extracellular, where they primarily function to maintain cellular integrity and prevent osmotic stress during the freezing and thawing processes[15]. Permeating cryoprotectants, such as DMSO and glycerol, diffuse across cell membranes, where they reduce ice crystal formation and minimize cellular injury by stabilizing intracellular structures during the freezing process[16]. DMSO has demonstrated a substantial ability to improve post-thaw cell viability and motility in cryopreserved sperm, exhibiting greater efficacy compared to non-permeating cryoprotectants[17]. DMSO modulates lipid membrane hydration dynamics, contributing to enhanced cellular survival rates by maintaining membrane stability during cryopreservation[16]. Non-permeating cryoprotectants, such as sucrose, preserve cellular integrity by preventing excessive dehydration and sustaining osmotic equilibrium without penetrating the cell membrane. The synergistic use of non-permeating and permeating agents has been shown to enhance cryopreservation efficacy, as evidenced by sucrose augmenting the protective effects of glycerol[18]. Permeating cryoprotectants are crucial for intracellular protection, while non-permeating agents serve a complementary function by supporting overall cryopreservation efficacy. This combined strategy enhances post-thaw cell viability and preserves functional integrity[15].

Reducing cryoinjury requires a complete understanding of the mechanisms underlying cellular damage during cryopreservation and the implementation of strategies to improve post-thaw viability. Identified approaches include the regulation of cellular processes and the application of cryoprotective agents to enhance cell survival[19]. Mesenchymal stem cells (MSCs) in the S-phase exhibit heightened susceptibility to cryoinjury, with an increased incidence of apoptosis resulting from DNA damage during the freezing process[20]. In addition to apoptosis, pathways like necroptosis and autophagy are involved in cryoinjury, indicating a complex interplay of various mechanisms[19]. Serum starvation before freezing can improve post-thaw viability by mitigating apoptosis in cell cycles that are particularly sensitive[20]. Zwitterionic betaine and trehalose have been demonstrated to prevent ice formation and enhance structural integrity during the cryopreservation of skin[21]. Sustaining a basal level of autophagy promotes the survival of spermatogonial stem cells after thawing, suggesting that autophagy is essential for cryoprotection[22].

The current application of cryoprotectants in cryopreservation predominantly involves conventional agents such as DMSO, glycerol, and ethylene glycol. While DMSO is extensively utilized, it is linked to cell cycle arrest and apoptosis[13]. Glycerol is effective for cryopreserving red blood cells. However, it can lead to haemolysis and cellular deformation. These adverse effects limit its broader application in cryopreservation[23]. Compounds such

as glycine and betaine have been recognized as biocompatible alternatives, effectively restoring electrostatic interactions within cells under stress. These properties contribute to their potential as protective agents in cryopreservation[24]. Elevated concentrations of cryoprotectants can induce cellular toxicity, thereby restricting their practical use in cryopreservation. This toxicity presents a challenge to optimizing cryoprotective agent formulations for effective preservation[25]. The efficacy of cryoprotectants varies considerably, requiring thorough assessment of their performance in maintaining cell integrity. This evaluation is essential for selecting the most suitable cryoprotective agents for specific preservation applications[23].

Table 1 provides a comparative overview of traditional and novel cryoprotectants used in sperm cryopreservation. Traditional cryoprotectants like DMSO, glycerol, and sucrose have been widely used but they face challenges such as cytotoxicity and osmotic stress. In contrast, novel alternatives, including amino acids, deep eutectic solvents, and cryoprotective polymers, offer reduced toxicity and enhanced cryoprotective efficacy, facilitating the way for safer and more effective cryopreservation strategies.

### *3.2. Effects of cryopreservation on sperm quality and the challenges of long-term sperm preservation*

Cryopreservation significantly impacts sperm motility, viability, DNA integrity, and acrosome function, with oxidative, osmotic, and thermal stresses playing a key role. These stressors contribute to cryodamage, resulting in a decline in sperm quality after thawing[26]. Cryopreservation frequently leads to reduced motility and viability, primarily due to oxidative stress caused by the generation of reactive oxygen species (ROS) during freezing. Elevated ROS levels can impair DNA integrity, which is essential for successful fertilization and proper genetic transmission[27]. Although acrosome integrity may be preserved, motility and viability are more significantly impacted by the conditions of cryopreservation[28]. The production of ROS during cryopreservation induces mitochondrial dysfunction, which further accelerates the decline in sperm quality[29]. Sperm undergo osmotic stress during freezing, which can negatively affect motility and viability. Rapid temperature fluctuations during thawing introduce further stress, amplifying the combined effects of osmotic and oxidative stresses[30]. Conversely, some studies indicate that specific cryoprotectants may alleviate these stresses, although their effectiveness can vary, and they may also pose a risk of toxicity[28].

Long-term cryopreservation of sperm involves various challenges that may impact sperm viability and subsequent fertility success. These challenges include the preservation of cellular integrity and the preservation of functional capabilities after thawing[31]. Sperm viability in long-term cryopreservation is affected by freezing techniques, cryoprotectant quality, and sperm biology. Although cryoprotectants like glycerol mitigate osmotic stress, they may not fully prevent membrane and DNA damage. Additionally, ROS can

**Table 1.** Traditional and novel cryoprotectants, and their characteristics and potential issues, and benefits.

| Category                    | Cryoprotectant                                                                        | Characteristics                              | Potential issues/Benefits                                                  |
|-----------------------------|---------------------------------------------------------------------------------------|----------------------------------------------|----------------------------------------------------------------------------|
| Traditional cryoprotectants | Dimethyl sulfoxide (DMSO)                                                             | Permeating, widely used                      | High cytotoxicity, apoptosis, gene expression alterations                  |
|                             | Glycerol                                                                              | Permeating, common in sperm cryopreservation | May cause osmotic stress, requires controlled removal post-thaw            |
|                             | Ethylene glycol                                                                       | Permeating, used in some applications        | Lower toxicity than DMSO, but not always optimal for sperm                 |
|                             | Sucrose                                                                               | Non-permeating, used as an osmoprotectant    | Helps prevent excessive dehydration, often combined with permeating agents |
|                             | Trehalose                                                                             | Non-permeating, cryoprotective sugar         | Maintains membrane integrity, reduces oxidative damage                     |
| Novel cryoprotectants       | Amino acids ( <i>L</i> -proline, <i>L</i> -carnitine, <i>L</i> -methionine sulfoxide) | Natural, biocompatible                       | Lower cytotoxicity, supports cell metabolism                               |
|                             | Deep eutectic solvents                                                                | Alternative solvent system                   | Reduced toxicity, potential for improved cryoprotection                    |
|                             | Cryoprotective polymers ( $\gamma$ -poly (glutamic acid))                             | Ice recrystallization inhibition             | High post-thaw recovery rates                                              |
|                             | FucoPol (Polysaccharide-based cryoprotective agent)                                   | Natural source of cryoprotection             | Forms protective gels, reduces ice damage                                  |
|                             | Ionic liquids                                                                         | Non-traditional, emerging alternative        | Lower toxicity, may enhance cryosurvival                                   |

induce DNA fragmentation and functional impairment, highlighting the necessity of antioxidants in cryopreservation protocols[32]. Research suggests that although motility and survival rates may remain consistent over 20-27 years, there is a notable increase in acrosome damage and a reduction in the sperm's ability to bind to oviductal cells[33]. Post-thaw evaluations typically show that approximately 50% of sperm maintain viability, with motility being a crucial determinant for successful fertilization[34]. Fertility outcomes indicate a cumulative live birth rate of 58.8% among couples utilizing cryopreserved sperm, suggesting that despite the challenges, successful outcomes are attainable[35].

Despite advancements in cryopreservation methods, the risk of sperm damage during freezing and thawing persists, emphasizing the need for ongoing research to optimize protocols and ensure optimal viability and functionality, particularly in ART[36].

### 3.3. Emerging alternatives: Towards less toxic cryoprotectants

Innovations in polymer-based and sugar-derived cryoprotectants have notably advanced cryopreservation, providing safer and more efficient alternatives to conventional approaches[37]. Cryoprotective polymers, including those containing DMSO-like moieties, demonstrate high post-thaw recovery by preventing ice formation and disrupting water's hydrogen-bonding networks[38]. Modified polymers, such as hydrophobically modified  $\gamma$ -poly(glutamic acid) (PGA) in combination with trehalose, markedly enhance red blood cell (RBC) cryosurvival, achieving survival rates of up to 90%[39]. Trehalose is well-known for its outstanding stabilization properties, which enhance the cryopreservation of proteins and cells. Additionally, the glycosylation of drug carriers with trehalose improves their biocompatibility and stability[40]. Polysaccharides such as FucoPol is a natural source of pure monosaccharides (*L*-fucose, galactose, glucose and glucuronic acid) and pectin provide effective cryoprotection by promoting

gel formation and attenuating supercooling, thereby reducing ice-induced damage during freezing[41].

Antioxidants are essential in lessening oxidative stress, which results from an imbalance between free radicals and antioxidants in the body. This imbalance can contribute to the development of various health problems, including chronic diseases and skin conditions[42]. Nutrient antioxidants, such as vitamins C and E, along with phytochemicals like flavonoids and carotenoids, are crucial in combating oxidative stress[43]. Enzymes like superoxide dismutase and catalase play a vital role in detoxifying free radicals, thereby protecting cells from oxidative damage. These enzymes contribute to the body's defence mechanisms against oxidative stress[44]. Foods abundant in antioxidants, including berries, nuts, and green leafy vegetables, are associated with a reduced risk of chronic diseases. Antioxidants have been linked to lower incidences of cancer, cardiovascular diseases, and neurodegenerative disorders[45]. Natural antioxidants, such as curcumin and garlic, have demonstrated protective effects against oxidative damage. These compounds help alleviate symptoms of skin conditions, including dermatitis and photoaging, by mitigating oxidative stress in skin cells[46].

Vitrification techniques and cryoprotectant-free methods mark significant progress in cryopreservation, improving the viability of biological samples while reducing cellular damage[47]. Vitrification, which utilizes rapid cooling to avoid ice formation, has proven to be more effective than conventional slow freezing techniques[48]. Vitrification enables rapid cooling, effectively preventing the formation of ice crystals that can cause cellular damage. This process preserves cell integrity by avoiding ice-induced stress during freezing[49]. Sustaining physiological temperatures throughout the process helps preserve the structural integrity of oocytes. This approach minimizes thermal stress and contributes to the overall stability of the cells during preservation[50]. Vitrification has been demonstrated to improve the viability of bovine oocytes and embryos more effectively than traditional slow freezing methods;

this technique enhances survival rates by minimizing ice-related damage[51]. The Direct Dropping Method involves immersing spermatozoa in a cooling agent, such as liquid air, which reduces contamination risks while enhancing motility and viability. Cryoprotectant-free methods are often more cost-effective and easier to implement in clinical environments[52]. Numerous studies have emphasized the advantages of maintaining physiological temperatures and using DMSO-free solutions, which reduce cytotoxicity and enhance preservation outcomes for oocytes and spermatozoa.

The cryopreservation method has a number of benefits that include good post-thaw recovery, increased survival, low ice-induced damage, high biocompatibility, and its cost-effectiveness. Nevertheless, there are some limitations such as possible cytotoxicity, complicated application, insufficient long-term studies, requirement for specialized expertise, and regulatory issues.

#### 4. Future directions and recommendations

Recent progress in multiomics integration and the development of personalized protocols is facilitating enhanced outcomes in the field[53]. The implementation of tailored cryopreservation protocols can significantly improve sperm viability following the thawing process. Personalized approaches optimize cellular integrity and functionality, contributing to better post-thaw recovery and fertilization potential[54]. Protocol modifications should be informed by variables such as chronological age, overall health condition, and specific reproductive health concerns to optimize cryopreservation outcomes[55]. Adjusting cryoprotectant concentrations and cooling rates according to individual patient profiles can enhance the efficacy of cryopreservation and improve post-thaw outcomes[56]. Multiomics approaches, including transcriptomics, proteomics, and metabolomics, have identified key genes like Collagen Type XI Alpha 1 Chain (*COL11A1*) and Nicotinamide Nucleotide Transhydrogenase (*NNT*) affected by cryopreservation. These biomarkers serve as indicators of sperm quality and aid in developing personalized treatment strategies[57]. Further in-depth research is essential to fully understand the long-term impact of cryopreservation on sperm functionality and its implications for reproductive outcomes[58]. Research efforts should prioritize investigating the underlying mechanisms of cryoinjury and the development of innovative cryoprotectants to enhance cryopreservation outcomes[56]. Future research should focus on identifying biomarkers to improve cryopreservation techniques and addressing critical gaps in the current understanding of the field[59].

#### 5. Limitations of the study

The paper offers significant insights of sperm cryopreservation,

its limitations include a restricted emphasis on existing techniques, the absence of longitudinal data, inadequate investigation into inter-individual variability and cryoprotectant-associated toxicity, overgeneralization of results, and an evident need for further research into potential biomarkers.

#### 6. Conclusions

Sperm cryopreservation remains a pivotal technique for preserving male fertility, particularly in the context of medical treatments that pose risks to reproductive health. Despite the established methods, challenges persist, particularly concerning DNA integrity and the viability of sperm post-thaw. The exploration of alternative cryoprotectants and innovative techniques, such as vitrification and cryoprotectant-free methods, has shown promise in enhancing sperm survival and functionality. Moreover, the integration of multiomics approaches to identify biomarkers for sperm quality is a significant advancement that can lead to personalized cryopreservation protocols tailored to individual patient needs. Future research should focus on elucidating the mechanisms of cryoinjury and developing novel cryoprotectants to mitigate cellular damage during the freezing process. By addressing these challenges and leveraging new technologies, the field of sperm cryopreservation can improve outcomes for men facing fertility issues, ultimately contributing to better reproductive health and success rates in ART.

#### Conflict of interest statement

The authors declare that there is no conflict of interest.

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#### Authors' contributions

Varaganti Pravardhan contributed to the conception or design of the work, drafting of the work, approval of the final version of the manuscript, and agreed on all aspects of the work. Nancy Nair contributed to the literature review, revision of the manuscript for important intellectual content, approval of the final version of the manuscript, and agreed to all aspects of the work. Varaganti Venkata Sai Suvardhan contributed to the literature review, revision of the manuscript for important intellectual content, approval of the final version of the manuscript, and agreed to all aspects of the work. Trupti Balwir contributed to the review and revision of the manuscript for important intellectual content, approval of the final version of the manuscript, and agreed to all aspects of the work.

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