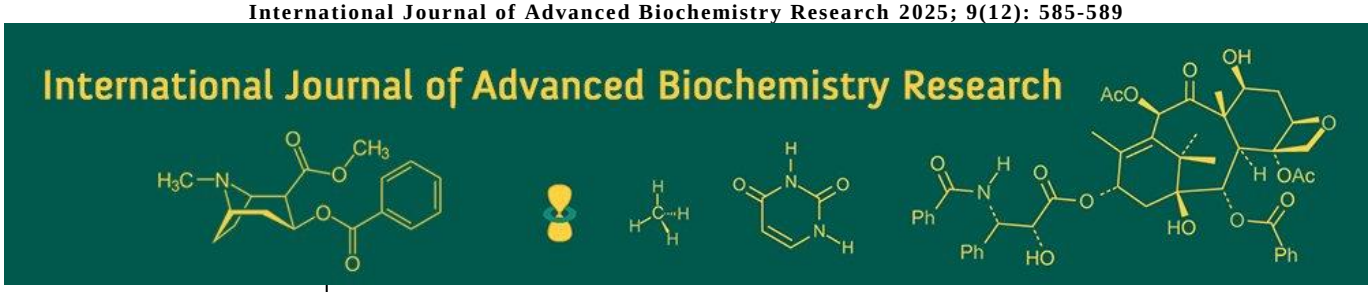

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Freeze-drying of sperm: A promising alternative to cryopreservation

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Abstract
The long-term preservation of mammalian spermatozoa is essential for assisted reproduction, livestock breeding, genetic resource conservation and protection of endangered species. Although cryopreservation in liquid nitrogen (LN_2) is a conventional method, its dependence on continuous LN_2 supply, high maintenance costs and vulnerability to natural disasters highlight the need for robust, low-cost alternatives. Freeze-drying (lyophilization) has emerged as a promising LN_2-independent preservation strategy, enabling stable storage of sperm at room or refrigeration temperatures. Initially used for microorganisms, freeze-drying has been successfully applied to mammalian sperm, with the first live offspring produced via ICSI using freeze-dried mouse sperm in 1998. The technique involves controlled freezing, primary sublimation and secondary desorption to produce a dry, stable product. Protective media containing Tris, EDTA/EGTA or trehalose play critical roles in maintaining DNA and chromosomal integrity. Although freeze-dried sperm lose motility, their genomic and oocyte-activating capacities remain intact, enabling successful fertilization through ICSI. DNA integrity assessed using AO, SCD and TUNEL assays shows no significant increase in fragmentation compared to fresh sperm. Freeze-dried sperm offer major advantages, including simplified storage and transport, reduced risk of pathogen transmission, suitability for remote regions and disaster-prone areas and long-term genetic banking. Future advancements should focus on improving post-rehydration viability, optimizing FD media and expanding the application of freeze-dried sperm for wildlife conservation and global biobanking initiatives.

Keywords: Freeze-drying, mammalian sperm, cryopreservation

Introduction

Preservation of mammalian spermatozoa: need for alternative approaches

The preservation of mammalian sperm, oocytes and embryos is crucial for infertility treatments, the conservation of genetically modified strains, livestock production and the conservation of endangered species (Kamada *et al.*, 2025) [16]. Cryopreservation in liquid nitrogen (LN_2) has long been the standard method for sperm storage. However, this technique has a several limitations like continuous LN_2 supply, high maintenance costs and dependency on specialized equipment (Dickey *et al.*, 2006) [7]. In addition, during natural disasters such as earthquakes or typhoons, valuable genetic resources stored in LN_2 may be lost due to interruptions in supply.

Although cryopreservation allows long-term storage at -196 °C, the ability to preserve sperm at higher, ideally ambient, temperatures is highly desirable. Such approach would reduce costs and space requirements, simplify sample transport, minimize viral contamination risks and provide an option for regions where LN_2 is scarce (Comizzoli *et al.*, 2022b; Loi *et al.*, 2013) [4, 27]. These challenges have driven interest in alternative storage technologies that enable preservation at supra zero temperatures.

Freeze-drying (lyophilization) offers one such promising approach. It converts samples into a stable dry state through controlled primary and secondary drying involving phase transitions. Initially it was used for preservation of microorganisms such as yeast and bacteria, this technique has now been explored for mammalian sperm (Vigneshwaran and Meganath, 2025) [36]. Although earlier attempts using ethanol, salt or Ficoll proved ineffective for long-term sperm preservation, freeze-drying has shown encouraging potential for LN_2-free storage. Importantly,

dependence on LN_2 makes cryogenic systems fragile and disaster-prone. Developing robust, low-cost and disaster-resilient preservation strategies independent of LN_2 is an urgent global priority.

Learning from nature's tricks

Nature provides valuable insights into dry preservation. Many seeds and anhydrobiotic organisms can survive extended periods in a desiccated state (Crowe and Crowe 2000) [5]. These biological adaptations inspire research into dry preservation strategies for spermatozoa. It offers a foundation for developing long-term, LN_2-independent storage solutions for gametes and embryos preservation.

Various drying technologies have been explored to preserve cells in a dry state, including lyophilization (freeze-drying) (Kawase *et al.*, 2005) [20], convective drying (Storey *et al.*, 1998) [34], spin drying (Chakraborty *et al.*, 2011) and microwave-assisted drying (Chakraborty *et al.*, 2008) [2, 3]. Among these, lyophilization remains the most effective and widely used technique for stabilizing perishable materials, protein pharmaceuticals and biological specimens for long-term storage and transport.

What is lyophilization or freeze-drying

Lyophilization, also known as freeze-drying, is a dehydration process in which frozen material is dried through the sublimation of ice, allowing a direct transition from the solid (ice) to the vapor phase without passing through the liquid state (Gil *et al.*, 2014) [11]. This technique has gained significant attention for sperm preservation due to its potential for long-term genetic storage without the need for liquid nitrogen (Kaneko *et al.*, 2014) [19]. The earliest attempts to preserve cells by dehydration were carried out using microorganisms such as bacteria, viruses and fungi (Flosdorf and Kimball, 1939) [10]. By the late 1950s, lyophilization found broad applications in the food industry for the preservation of products like milk, eggs, yeast, coffee, soups and juices. Recently, the application of freeze-dried sperm preservation has expanded toward pioneering biobanking concepts, such as storing genetic material in lunar lava tubes to ensure the long-term conservation of biodiversity (Wakayama *et al.*, 2017) [37].

Freeze-drying media

The freeze-drying (FD) medium is crucial for maintaining sperm DNA integrity and ensuring stability after rehydration. It should be a balanced salt solution with suitable osmotic pressure and pH, providing a stable matrix for drying and rehydration (Nakai *et al.*, 2007) [30]. FD media containing NaCl and high Tris-HCl buffer (100 mM) effectively reduced DNA fragmentation in freeze-dried spermatozoa (Kusakabe *et al.*, 2008) [26]. As sperm membrane integrity is not required for fertilization via intra cytoplasmic sperm injection (ICSI), cryoprotectants are unnecessary in FD media.

Current scenario and improvements in FD media

The first successful production of mouse offspring using freeze-dried sperm through ICSI was achieved using a medium supplemented with 10% fetal calf serum (FCS) (Wakayama and Yanagimachi, 1998) [38]. Subsequent modifications to FD media have focused on improving DNA stability and chromosomal integrity. For instance, the incorporation of ethylene glycol-bis (β-aminoethyl ether) tetra-acetic acid (EGTA) inhibited endonuclease activity (Kusakabe *et al.*, 2001) [25], while its substitution with EDTA maintained calcium chelation and enhanced chromosomal stability (Kaneko and Nakagata, 2006) [18]. A slightly alkaline environment (pH 8.0) was also found optimal for preserving chromosome integrity and developmental potential of mouse spermatozoa (Kaneko and Nakagata, 2006) [18]. Moreover, eliminating NaCl and increasing Tris-HCl concentration in the FD medium further minimized DNA fragmentation (Kusakabe *et al.*, 2008) [26]. In bovine sperm, the addition of trehalose along with FCS offered superior DNA protection compared to FCS alone (Martins *et al.*, 2007) [28]. A simple yet effective formulation consisting of 10 mM Tris and 1 mM EDTA at pH 8.0 has been proposed to protect sperm DNA by inactivating DNases under mildly alkaline conditions.

Trehalose

Trehalose is a natural disaccharide composed of two glucose molecules linked by a 1-1 glycosidic bond and occurs at high concentrations in many desiccation-tolerant organisms. It plays a critical role in anhydrobiosis, the ability of certain organisms to survive extreme dehydration, sometimes comprising up to 20% of the dry weight. Trehalose promotes the formation of an amorphous glassy matrix, inhibits crystallization, and interacts with cellular structures to stabilize them during drying (Koster *et al.*, 2000; Brogna *et al.*, 2020) [1, 24]. This glassy state, characterized by very high viscosity, physically shields biomolecules, including those in cell membranes, from chemical, biological and physical stresses, thereby preventing deterioration and cell death (Crowe *et al.*, 2004) [6].

Process of lyophilization for spermatozoa

1. Sample preparation with FD media
2. Freeze Drying Process: includes Freezing, Primary drying, Secondary drying (Keskintepe and Eroglu, 2015) [23]

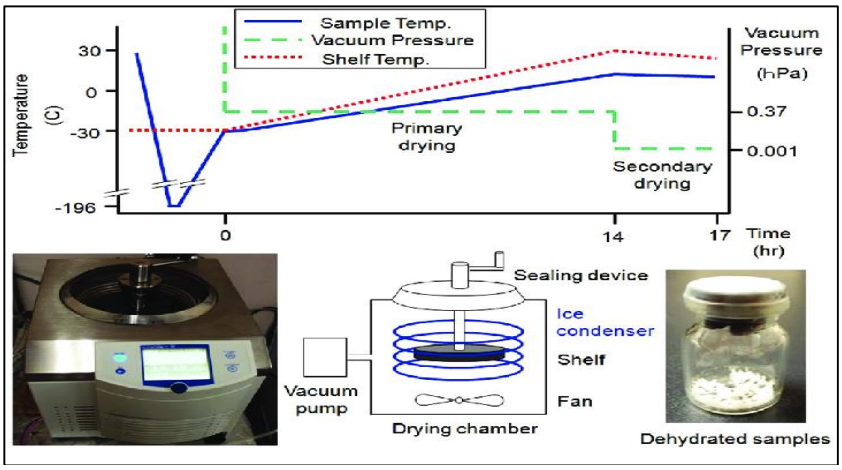
Most important factors that affect the FD protocol are the vacuum pressure, temperature drying period and freeze-drying solution (Hochi *et al.*, 2011) [15].

Primary drying

In the primary drying step of freeze-drying, the pressure inside the machine is lowered and gentle heat is applied to start turning the ice directly into vapor. Before this, the sample is frozen by cooling it below its eutectic temperature so that the water turns into ice and separates from the other substances. Then, a vacuum is created to reduce the water pressure below its triple point, which allows the ice to change directly into vapor without melting. This process, called sublimation. For sublimation to work well, there must be a difference in temperature between the drying chamber and the condenser. The condenser is kept much colder so it can collect the water vapor and turn it back into ice. Primary drying continues until all the free ice is removed, after completion of primary drying about 8-10 percent moisture remains inside the sample. The next step, called secondary drying, removes the remaining bound water by further heating under vacuum. After this, the dried sample can be safely stored at room temperature and easily restored by adding water. (Keskintepe and Eroglu, 2015) [23].

Secondary drying: During the secondary drying stage, the residual bound water is eliminated through desorption to

maintain the stability of the sample. The process involves gradually heating the material under an ultra-low vacuum (around 0.001 mbar), which promotes the removal of the remaining water molecules. This step, typically conducted for 3-6 hours at around 30°C, further reduces the moisture content to below 1%, achieving optimal dryness of the material (Keskintepe and Eroglu, 2014) [22].



Kinetics of Vacuum Pressure and Shelf Temperature Programmed for a SpermFreeze-drying Protocol in a Freeze-dryer Machine (ALPHA 2-4 LSC) (Hochi *et al.*, 2011) [15].

Sample storage

Freeze-dried spermatozoa can remain viable for extended periods, depending on storage temperature. Wakayama and Yanagimachi (1998) [38] reported that room-temperature storage-maintained sperm viability for only up to one month, whereas storage at 4°C or 25°C for three months did not affect embryonic development. Kawase *et al.* (2005) [20] found higher blastocyst rates after six months of storage at -80°C compared to 4°C, suggesting that preservation below -80°C could last over a century. However, Kaneko and Nakagata (2006) [18] identified 4°C as an optimal and economical temperature for long-term preservation and transport, a finding supported by Kaneko and Serikawa (2012) [17], who achieved successful offspring from sperm stored at 4°C for three years.

Rehydration of Freeze-Dried Spermatozoa

Rehydration, or reconstitution, involves restoring freeze-dried sperm to its original state by adding pure water equal in volume to the initial sperm suspension. Although rehydrated spermatozoa are non-motile but their DNA integrity and oocyte-activating factors remain intact, allowing successful use in assisted reproduction (Gil *et al.*, 2014) [11].

Assessment of Sperm DNA Integrity

Sperm DNA and chromatin integrity can be evaluated using several techniques, including the acridine orange test, Sperm Chromatin Dispersion (SCD) test and TUNEL assay. The acridine orange test differentiates intact double-stranded DNA, which fluoresces green, while the denatured or damaged DNA appears orange. The SCD test is based on the principle that sperm with intact DNA form large halos of dispersed chromatin after acid denaturation and lysis, while fragmented DNA produces small or no halos; normal DNA integrity is indicated by a DNA fragmentation index below 15% (Fernández *et al.*, 2005; Olaciregui and gil, 2017) [9, 31]. The TUNEL assay, considered more sensitive for lyophilized sperm, it detects single and doublestrand DNA breaks by labeling free 3′-OH ends with nucleotides via terminal deoxynucleotidyl transferase (TdT). Fragmented DNA fluoresces green and intact DNA blue under fluorescence microscopy or flow cytometry, with normal fragmentation levels below 10-20% (Martins *et al.*, 2007) [28]. Overall, studies indicate no significant difference in DNA damage between fresh and freeze-dried sperm samples (Kawase *et al.*, 2009; Hara *et al.*, 2011) [13, 21].

Intracytoplasmic Sperm Injection (ICSI)

Intracytoplasmic sperm injection (ICSI) is an assisted reproductive technique in which a single sperm is directly injected into an oocyte by penetrating the zona pellucida and oolemma of oocyte. The first successful ICSI was reported in sea urchin eggs by Hiramoto in 1962 [14], and the first ICSI-derived mammalian offspring, a calf, was produced in 1990 (Goto *et al.*, 1990) [12]. Freeze-dried sperm lose motility and show structural alterations, making natural fertilization impossible; however, ICSI can overcome these limitations.

Key Considerations for ICSI

The oocyte should be held with the polar body positioned at 6 or 12 o'clock. The injection needle should be inserted at the 3 o'clock position. Oocytes with a larger cytoplasmic volume have a higher chance of developing into blastocysts (Dumoulin *et al.*, 2001) [8]. Success rates can be improved by physical manipulations such as acrosome removal, sperm immobilization or crushing the sperm tail and membranes (Keskintepe and Eragulu, 2014) [22].

Advantages and applications of freeze-dried semen

Freeze-drying of spermatozoa offers a simple, safe and economical alternative to cryopreservation for long-term storage and transport without the need for liquid nitrogen (Kamada *et al.*, 2025) [16]. It enables convenient preservation of numerous genetic strains and endangered species in "sperm books," minimizing space, cost and maintenance requirements. The technique has even produced offspring after long-term storage in international space station, demonstrating its durability and global potential. Moreover, since many bacteria and viruses cannot withstand

desiccation, the method also reduces the risk of disease transmission (Tedder *et al.*, 1995; Russel *et al.*, 1997) [35].

Critical Factors Influencing Freeze-Drying Success

The effectiveness of sperm freeze-drying depends on protective additives, storage conditions and rehydration methods. Additives like trehalose preserve acrosomal integrity (Men *et al.*, 2013), while chelating agents such as EDTA and EGTA protect DNA from enzymatic damage (Kusakabe*et al.*, 2001) [25]. Proper storage, typically at 4°C, maintains long-term stability, with lower temperatures (-80°C) potentially extending preservation for decades (Kaneko and Serikawa, 2012) [17].

Conclusions and future prospects

Freeze-drying is a promising alternative to traditional cryopreservation, allowing stable preservation of sperm DNA and genetic material without the need for liquid nitrogen. It enables easy storage and transport at room or refrigerated temperatures and holds significant potential for wildlife conservation. Future research should focus on improving sperm motility and viability after rehydration, optimizing preservation media and protocols, and establishing long-term storage strategies to enhance the utility of freeze-dried sperm in reproductive science and biodiversity preservation.

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