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Review on advances in sperm preservation: Freeze-drying as an alternative to cryopreservation

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Abstract

Sperm preservation is a vital component of assisted reproductive technologies (ART), livestock breeding programmes, and conservation efforts for endangered species. Although cryopreservation in liquid nitrogen (LN₂) remains the gold standard, it poses significant challenges, including prohibitive costs, contamination risks, and logistical complexities. In response, freeze-drying, or lyophilization, has emerged as a promising alternative, enabling long-term sperm storage at ambient temperatures while maintaining DNA integrity. This review provides an in-depth examination of the principles, benefits, and limitations of freeze-drying in comparison to conventional cryopreservation. We discuss key factors influencing the efficiency of freeze-drying, including the role of protective additives such as trehalose, EDTA (Ethylenediaminetetraacetic Acid), and EGTA (Ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid), optimal storage conditions, and precise rehydration techniques. Furthermore, we highlight successful applications of freeze-dried sperm across various species and outline future directions for optimising this technology in both veterinary and human reproductive medicine. By exploring the potential of freeze-drying, this review aims to contribute to the advancement of sperm preservation techniques and their applications in biomedicine and conservation.

Keywords: Sperm preservation, freeze-drying, lyophilization, cryopreservation, assisted reproductive technology, DNA integrity

Introduction

The use of artificial insemination (AI) with frozen-thawed semen in certain species, such as buffalo, is limited due to poor sperm survivability after thawing, resulting in significant sperm damage (Das, 2021) ^[3]. Cryopreservation, despite its widespread use, can cause substantial damage to spermatozoa, with approximately 40-50% of sperm being sensitive to freezing stress (Polge *et al.*, 1949) ^[20]. As an alternative, freeze-drying has emerged as a promising method, which involves removing water through sublimation, allowing for storage at 4°C or room temperature (Kaneko & Serikawa, 2012) ^[11]. This review assesses the potential of freeze-drying as a sperm preservation method, comparing its effectiveness with cryopreservation and discussing recent advancements in DNA protection techniques.

Cryopreservation: Current Status and Limitations

Cryopreservation involves cooling sperm to -196°C in liquid nitrogen, effectively halting metabolic activity and preserving sperm viability for extended periods. The addition of cryoprotectants, such as glycerol, plays a crucial role in mitigating ice crystal formation during the freezing process, which can cause significant damage to sperm cells (Polge *et al.*, 1949) ^[20]. However, cryopreservation has notable limitations that impact its practical application. The requirement for a continuous supply of liquid nitrogen poses significant logistical and financial challenges, particularly in regions with limited infrastructure. For instance, India alone consumes an estimated 40 million liters of liquid nitrogen annually for artificial insemination procedures, highlighting the scale of this issue (Das, 2021) ^[3]. Furthermore, the use of cryoprotectants can be toxic to sperm cells at high concentrations, potentially leading to DNA damage. Contamination risks also remain a concern, underscoring the need for stringent handling and storage protocols. Despite these challenges, cryopreservation allows for indefinite storage of sperm and maintains high embryo development rates, making it a valuable technique in assisted reproduction.

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Nevertheless, these limitations have driven the search for alternative sperm preservation methods that can offer comparable efficacy with reduced logistical and financial burdens.

Freeze-Drying (Lyophilization) as an Alternative Principles of Freeze-Drying

Freeze-drying, or lyophilization, is a sophisticated dehydration process that preserves biological materials by removing water through sublimation under vacuum conditions (Kwon *et al.*, 2004) ^[14]. The technique involves three critical phases: initial freezing of the sample, primary drying where frozen water sublimates directly from solid to vapor phase, and secondary drying to remove residual moisture. This method maintains the structural and functional integrity of biological specimens by avoiding the damaging effects of liquid water removal, making it particularly valuable for sperm preservation. The process requires precise control of temperature and pressure parameters to ensure optimal preservation while minimizing cellular damage. A key advantage of freeze-drying is its ability to stabilize biological materials at ambient temperatures, eliminating the need for continuous cryogenic storage (Kaneko & Serikawa, 2012) ^[11].

Historical Development of Freeze-Drying

The application of freeze-drying technology to sperm preservation has evolved through several key milestones. The foundational work began with early dehydration experiments on microorganisms (Flosdorf & Kimball, 1939) ^[4], leading to the first successful freeze-drying of fowl semen (Polge *et al.*, 1949) ^[20]. Subsequent advances included pioneering studies with human sperm (Sherman, 1954) and bull spermatozoa (Bialy & Smith, 1957) ^[1]. A major breakthrough occurred in 1998 with the birth of live offspring from freeze-dried mouse sperm, demonstrating the technique's reproductive potential (Wakayama & Yanagimachi, 1998) ^[25]. While the method requires ICSI for successful fertilization (Kwon *et al.*, 2004) ^[14], its ability to maintain sperm DNA integrity during storage at refrigerator temperatures (4°C) or even ambient conditions has established freeze-drying as a practical alternative to conventional cryopreservation. These developments have positioned lyophilization as a valuable tool for long-term preservation of genetic material across multiple species.

Advantages over Cryopreservation

Freeze-drying offers several benefits, including cost-effectiveness and logistical convenience. By eliminating the need for liquid nitrogen, it enables room-temperature storage and simplifies transportation. Furthermore, freeze-dried sperm shows reduced oxidative stress, with lower lipid peroxidation levels compared to cryopreserved samples, potentially preserving sperm quality (Shahba *et al.*, 2016) ^[23].

Limitations

A significant limitation of freeze-dried sperm is its inability to fertilize eggs naturally after rehydration, requiring the use of intracytoplasmic sperm injection (ICSI) for fertilization. Additionally, DNA fragmentation is a concern, although the use of chelating agents like EDTA or EGTA has been shown to help minimize this risk and preserve DNA integrity (Kaneko & Nakagata, 2006) ^[10].

Critical Factors Affecting Freeze-Drying Success

Protective Additives

Certain additives have been shown to enhance sperm preservation during freeze-drying. Trehalose helps maintain acrosome integrity (Men *et al.*, 2013) ^[16], while chelating agents like EDTA and EGTA protect against DNase-induced DNA damage (Kusakabe *et al.*, 2001) ^[13]. These additives play a key role in preserving sperm functionality and DNA integrity during the lyophilization process.

Storage Conditions

Optimal storage conditions are crucial for maintaining the integrity of freeze-dried sperm. Research indicates that storage at 4°C can provide long-term stability (Kaneko & Serikawa, 2012) ^[11], while even lower temperatures, such as -80°C, may further extend preservation duration, potentially for decades (Kawase *et al.*, 2007) ^[12].

Rehydration Techniques

Rehydrating freeze-dried sperm with pure water to the original suspension volume can help restore structural integrity (Gil *et al.*, 2014) ^[6]. Additionally, incorporating DNA protectants into the rehydration medium may further enhance fertilization potential (Olaciregui & Gil, 2017) ^[18].

Applications Across Species

Freeze-dried sperm has been successfully used to produce offspring in several species, demonstrating its potential as an alternative to conventional cryopreservation. Following the initial success with mice (Wakayama & Yanagimachi, 1998) ^[25], the technique has been effectively applied across diverse species including rabbits (Liu *et al.*, 2004) ^[15], rats (Hirabayashi *et al.*, 2005; Kaneko & Serikawa, 2012) ^[8, 11], primates (Sanchez-Partida *et al.*, 2008) ^[22], dogs (Olaciregui *et al.*, 2015) ^[19], cats (Ringleb *et al.*, 2011) ^[21], horses (Choi *et al.*, 2011; Olaciregui *et al.*, 2015) ^[2, 19], hamsters (Muneto & Horiuchi, 2011) ^[17], and boars (García *et al.*, 2014; Men *et al.*, 2013) ^[5, 11]. In livestock species, particularly bulls (Hara *et al.*, 2014) and buffalo (Shahba *et al.*, 2016) ^[7, 23], freeze-drying has shown promise, although blastocyst development rates can vary and DNA damage remains a concern in some cases. The technique has also been extended to wild animal conservation efforts (Kaneko *et al.*, 2014) ^[9], demonstrating its broad applicability across mammalian species. These successful applications highlight freeze-drying as a viable alternative to traditional sperm cryopreservation methods, particularly in situations where liquid nitrogen storage is impractical.

Future Perspectives

The applications of freeze-dried sperm are diverse and far-reaching. In human reproductive medicine, freeze-drying could simplify storage by eliminating the need for liquid nitrogen, although ICSI would still be required. In wildlife conservation, freeze-drying offers a practical solution for preserving genetic material from endangered species. Furthermore, research into sperm preservation techniques could have implications for space exploration, where genetic material needs to be stored for extended periods under extreme conditions (Kaneko *et al.*, 2014) ^[9].

Conclusion

Freeze-drying is a promising alternative to traditional cryopreservation for sperm preservation, offering benefits

like reduced costs and simplified storage. Although challenges such as ICSI dependency and DNA fragmentation remain, ongoing research into protective additives and storage protocols is improving its viability. Future studies should prioritize optimizing lyophilization for human clinical applications and exploring its potential in wildlife conservation, potentially transforming reproductive sciences and biodiversity preservation.

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