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Handle with Freeze: Role of cryoprotectants in semen cryopreservation

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Abstract

Sperm cryopreservation is essential for genetic conservation and artificial insemination, but freezing and thawing cause cellular damage such as ice crystal formation, osmotic stress, and membrane disruption. Cryoprotectants are crucial in reducing these injuries by stabilizing membranes, controlling osmotic balance, and limiting ice crystal formation. They are classified into permeating (e.g., glycerol, DMSO) and non-permeating (e.g., sucrose, trehalose) types, each acting through distinct protective mechanisms. Freezing methods, including slow, rapid and vitrification, depend on these agents for effective preservation. Despite their benefits, issues like toxicity and osmotic damage persist. Advances in novel cryoprotectants and optimized protocols aim to improve sperm survival and fertility outcomes.

Keywords: Sperm, cryoprotectant, semen cryopreservation

1. Introduction

Mammalian sperm were among the first cells to be successfully cryopreserved, and over the last seventy years, the use of frozen semen in artificial insemination has become a vital tool in animal agriculture, revolutionizing breeding practices and genetic management (Curry et al., 2007).Preserving animal semen through cryopreservation has transformed modern veterinary science by making artificial insemination more efficient, supporting the conservation of valuable genetics, and facilitating the global exchange of germplasm.. Cryopreservation is the process by which cells or tissues can survive at low temperatures with reduced or ceased of metabolic activity (Watson et al., 2000). During this process, cells undergo multiple forms of damage affecting both their structural and functional integrity, which ultimately reduces overall cell or tissue performance. Specifically, sperm cells subjected to freezing and thawing face significant stress, including the formation of ice crystals, osmotic imbalances, and membrane damage, all of which can compromise their viability and fertilizing capacity (Parks et al., 1992). These cryoinjuries mostly lead to loss of motility, plasma membrane functionality, and acrosome integrity of sperm (Watson et al.,2000). Hence cryoprotectants play a crucial role in the

cryopreservation medium by reducing the physical and chemical stresses that sperm cells experience during cooling, freezing, and thawing.

Types of cryoprotectant and their mechanism of action

Cryoprotectants are chemical agents added to semen before freezing to minimize cellular damage caused by low temperatures and ice formation. They act by stabilizing membranes, reducing ice crystal formation, and controlling osmotic changes.(Ozimic et al., 2023).Cryopreservation requires protection of intracellular structures and biomolecules, which is achieved through cryoprotective agents that act either by penetrating the sperm cell membrane to protect intracellular components or by remaining extracellular to safeguard the cell from osmotic and ice crystal related stress.

Cryoprotectants are classified into permeating (penetrating) and non-permeating/non penetrating types, each with distinct roles in protecting sperm cells during cryopreservation are summarized in figure.1. Permeating cryoprotectants are small, non-ionic, membrane-permeable molecules that act both intra- and extracellularly. Examples include glycerol (Polge et al., 1949), dimethyl sulfoxide (Lovelock and Bishop, 1959), ethylene glycol, and propylene glycol. These agents first enter the sperm cell and reduce intracellular water content by osmotically drawing water out, which equilibrates intracellular and extracellular concentrations (Amann, 1999). This lowers the freezing point inside the cell, decreases the likelihood of intracellular ice formation, and protects organelles and biomolecules from mechanical damage during freezing. Permeating cryoprotectants also interact with lipid bilayers and proteins, increasing membrane fluidity, stabilizing membranes, and preserving enzymatic activity to prevent protein denaturation (Holt, 2000).Non-permeating cryoprotectants, in contrast, do not enter the cell but act extracellularly by increasing osmotic pressure outside the sperm, drawing water out to reduce ice formation and prevent excessive shrinkage or swelling. They may also modify the plasma membrane or lower the freezing temperature of the medium (Amann, 1999). Examples include sugars such as sucrose and trehalose, and polymers like egg yolk and milk proteins. By acting outside the cell, non-penetrating cryoprotectants further stabilize membranes and mitigate oxidative stress caused by reactive oxygen species (ROS) (Ozimic et al., 2023).

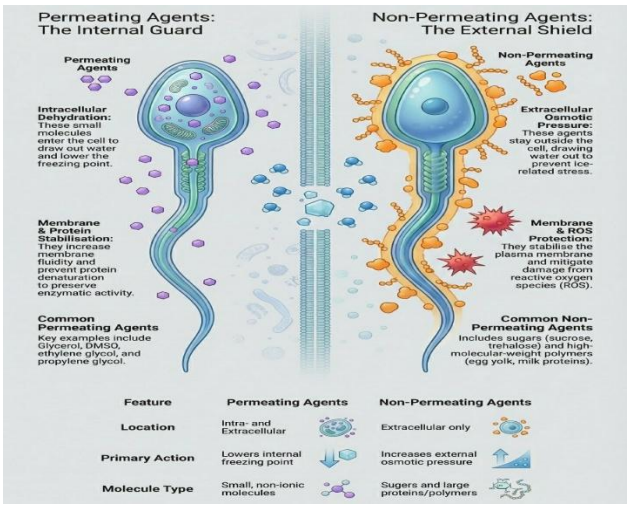

Figure 1: Action of cryoprotectants in semen freezing

Cryoprotectant-dependent methods of sperm freezing

Method	Cryoprotectant	Type	Key Action
Slow freezing	Glycerol	Permeating	Prevents intracellular ice
Rapid freezing	Dimethyl sulfoxide	Permeating	Fast penetration, partial protection
Ethylene glycol method	Ethylene glycol	Permeating	Low toxicity, rapid diffusion
Sugar-based method	Sucrose / Trehalose	Non-permeating	Osmotic dehydration
Vitrification	Mixed CPAs	Permeating + Non	No ice crystal formation

Merits and Demerits of lowering the freezing point in semen extenders

Lowering the freezing point of semen extender solutions using cryoprotectants offers several advantages. It reduces ice crystal formation inside and outside sperm cells, preventing physical damage to cell membranes and organelles, stabilizes cell membranes and proteins during freezing and thawing, and controls osmotic changes to avoid excessive cell shrinkage or swelling. This enhances sperm survival post-thaw, maintains motility, membrane integrity, and fertilizing capacity, and improves fertility rates in artificial insemination programs. Additionally, it enables long-term storage and global transport of semen, supports genetic improvement, rare breed and wildlife conservation, and reduces the need for live animal transport. However, there are some demerits associated with cryoprotectants. Permeating cryoprotectants like glycerol or DMSO can be toxic at high concentrations, and osmotic stress during addition or removal can damage sperm cells if not carefully managed.

Future Prospects of cryoprotectants in semen preservation

Future developments in cryoprotectants for semen preservation focus on designing novel, less toxic alternatives, such as antifreeze proteins, plant-derived compounds, and synthetic polymers, to enhance sperm survival and fertility outcomes. Nanotechnology is being applied to improve the delivery and reduce toxicity of cryoprotectants, while vitrification techniques aim for ice-free preservation. Combining cryoprotectants with antioxidants and membrane-stabilizing agents helps reduce oxidative stress during freezing and thawing. Advances in genomics and proteomics allow the development of species-specific cryoprotectant systems, and AI-driven predictive modeling enables precise optimization of freezing protocols. Together, these innovations aim to maximize fertility rates, improve breeding efficiency, and support long-term genetic conservation..

Conclusion

Cryobiology is increasingly important in veterinary science, especially for sperm preservation and global genetic exchange. Key challenges remain, including understanding cryoprotectant mechanisms, cellular interactions, and potential toxicity. Optimizing cryoprotectant type and concentration, along with precise cooling and freezing rates, is essential to maximize sperm survival, motility, and fertilizing capacity. While cryoprotectants effectively protect sperm by reducing ice formation, stabilizing membranes, and regulating osmotic changes, species variability and improper protocols can still compromise outcomes. Continued research on combining permeating and non-permeating agents, antioxidants, and equilibration strategies will enhance post-thaw viability. Modern semen cryobanking holds great promise for breeding, conservation, and artificial insemination programs, but standardization of cryoprotectant use is critical for consistent success

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