



Review article

Technological advances in chicken semen cryopreservation: Impacts on sperm quality, freezability and fertility

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Abstract

To facilitate genetic diversity preservation and conservation initiatives, poultry semen cryopreservation remains as a critical technology in avian reproduction. Despite notable achievements in assisted reproductive technologies in the species in the past decades, the biochemical and physiological traits of chicken semen pose unique challenges for cryopreservation. This review examined the factors influencing the quality, fertility, and freezability of cryopreserved chicken semen while emphasizing on the role of proteins, antioxidants, and cryoprotectants like ethylene glycol and dimethylacetamide in maintaining sperm integrity. The fundamentals of cryopreservation, including important procedures like collection, dilution, cooling, and thawing were also discussed alongside advances in current techniques such as slow freezing and vitrification, and novel approaches like microfluidics and nanoparticle-based cryoprotection. The effects of lipid peroxidation and oxidative stress on sperm structure and DNA integrity during freezing was also elaborated, suggesting new cryoprotectants and antioxidants as mitigating measures. Breed-specific variations and environmental factors are also explored, underscoring the need for customized strategies to maximize cryopreservation results. The analysis also looked at promising technologies for improving cryopreservation outcomes such as artificial sperm reservoirs. This article also highlights the significance of ongoing research to improve the dependability and accessibility of semen cryopreservation for both commercial and conservation purposes, while addressing contemporary issues like standardization and ethical considerations. In conclusion, this comprehensive review of existing knowledge and emerging trends provides a roadmap for transformative research and development breakthroughs in poultry semen reproductive technologies.

Keywords: Chicken semen, Cryoinjury, Cryopreservation, Fertility, Freezability, Oxidative stress, Sperm quality

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INTRODUCTION

Sperm cryopreservation represents a fundamental step in assisted reproductive technologies (ARTs) across various animal species, including fishes (Torres et al., 2016), birds (Nizam and Selçuk, 2021; Mehaisen et al., 2022), and mammals (Moreira et al., 2022). Cattle breeding programs, for instance, almost exclusively rely on cryopreserved semen from superior males for their artificial insemination practices to optimize reproductive outcomes and genetic gains (Najafi et al., 2020; Oldenhof et al., 2021). Beyond livestock production, cryopreservation offers a strategic tool for safeguarding the germplasm of genetically valuable animals and endangered species, enabling the implementation of other ARTs such as artificial insemination and in vitro fertilization (Barbas and Mascarenhas, 2009; Perez-Patiño et al., 2019; Ugur et al., 2019; Peris-Frau et al., 2020).

While the relatively advanced method of semen freezing has been extensively utilized in various mammalian species such as pigs, cattle, and sheep, the developed techniques cannot be directly adapted to poultry species due to fundamental differences in avian reproductive biology. Unlike mammals, the cryopreservation of oocytes or embryos in birds is particularly difficult due to the structural and physiological complexity (Zong et al., 2023). Therefore, sperm cryopreservation has become a pivotal focus for maintaining genetic diversity in both commercial poultry breeds and endangered wild bird populations. Despite significant challenges in the development and optimization of efficient avian sperm cryopreservation methods, there is a growing demand for this technology for both agricultural and conservation contexts.

Freezability is the capacity of sperm cells to tolerate freezing and thawing without major loss of key functions. These functions include sperm viability, motility, plasma membrane functionality and integrity, acrosome integrity, mitochondrial integrity, DNA integrity and fertilizing ability after cryopreservation (Blesbois et al., 2005; Long, 2006; Hamad et al., 2023; Najafi et al., 2024; Simsek et al., 2025). In poultry, low freezability is a main reason frozen semen often gives much lower fertility than fresh semen (Long, 2006; Mocé et al., 2010; Elomda et al., 2024; Zong et al., 2024). The ability to maintain chicken sperm fertility after cryopreservation was first demonstrated in 1949 by Polge and colleagues (Polge et al., 1949). Since then, various techniques for cryopreserving chicken semen have been explored across different countries, yet the current technologies are either technically not yet fully mature or yield inconsistent results. This unreliability or unpredictability in fertility rates, therefore, limit the adoption of chicken semen cryopreservation in commercial poultry industry. While some methods demonstrate promising results in terms of maintaining sperm viability, key parameters including post-thaw vitality and fertilization rates for cryopreserved avian semen remain suboptimal (Blesbois et al., 2007). In addition, the cryopreservation process itself exerts significant stress on the spermatozoa due to temperature variations, osmotic fluctuations, and intracellular ice crystal formation. Taken altogether, these factors compromise the structural integrity and physiological function of the sperm affecting its capacity to achieve successful fertilization (Braun et al., 1994; Suzuki et al., 2007). Aside from these established challenges, the complex sperm-freezing process entails several critical steps which must be carefully optimized, such as selection of suitable males, semen collection, quality assessment, semen dilution, addition of cryoprotectants (CPAs), packaging, freezing, thawing, and post-thaw evaluation (Svoradová et al., 2021; Yáñez-Ortiz et al., 2022).

This paper explores the current landscape of avian sperm cryopreservation research. An overview of the protocols currently employed at each stage of the cryopreservation process was provided, with emphasis on semen extenders and cryoprotectants currently used. Moreover, the implications of cryopreservation in sperm structure and function were also examined focusing on critical factors such as freezability, post-thaw quality, and fertility. In addition to evaluating existing methods, innovative experimental approaches for improving chicken sperm

cryopreservation outcomes were also highlighted at the later part of this review, all aimed to overcome the present limitations in avian reproductive biotechnology.

METHODOLOGY AND SEARCH STRATEGY

For this review, we searched major databases like PubMed, Scopus, PubMed Central and Google Scholar from the beginning of the database to the last search date and we did limit the search to English languages. The search strategy employed combinations of the following keywords: avian spermatozoa, semen cryopreservation, cryotolerance, sperm quality, poultry fertility, cryoprotectants, extenders, and freezing procedure. Peer-reviewed original research, meta-analyses, and high-impact reviews published in English, with a primary focus on avian species were included in this study. We excluded studies focusing solely on mammalian cryopreservation without comparative value, non-peer-reviewed reports, and studies where the methodology was not clearly defined.

BIOLOGY OF CHICKEN SEMEN

Chicken semen is primarily composed of high sperm concentrations usually between 3 and 8 billion sperms per milliliter (mL), essential for successful fertilization (Santiago-Moreno and Blesbois, 2020) and seminal plasma, which contains various biochemical components such as carbohydrates, lipids, amino acids, hormones, and proteins, which play roles in metabolism, immunity, and sperm motility (Santiago-Moreno and Blesbois, 2020). Compared to some animals, like humans and cows, chicken spermatozoa have a thinner tail and a comparatively lower surface area to volume ratio (Mohammad et al., 2021). The spermatozoa across various species exhibit a shared structural design which consist of the head and tail. Three parts make up the tail: the mid-piece, the principal-piece, and the end-piece (Zong et al., 2023). Avian spermatozoa vary greatly in length, ranging from 30 to 300 μm , and sperm heads vary in length from 11 to 21 μm . The chicken sperm's head measured roughly 12.5 μm and had a slight cylindrical curvature. The conical acrosome on top of the cranium measures roughly 2.5 μm . The primary component is roughly 90 μm , the mid-piece is roughly 4.5 μm , and the mitochondrial matrix is uniformly dense (Xia et al., 1988). Compared to chicken sperm, human and bull sperm typically have larger, oval heads (Zhu et al., 2022; Wiebke et al., 2022). The tail of chicken sperm is longer than that of other species, measuring roughly 90 μm (Grigg and Hodge, 1949; Xia et al., 1988).

Chicken semen contains a complex array of proteins and peptides. Labas et al. (2015) identified 1165 proteins in chicken semen, primarily involved in oxido-reduction mechanisms, energy processes, proteolysis, and protein localization. Specific proteins such as VMO1, AvBD10, and AvBD9 were identified, with variations linked to fertility levels (Labas et al., 2015). Serine ethanolamine phosphodiester is a major component in chicken semen, analogous to glycerol 3-phosphorylcholine in mammals (Burt and Chalovich, 1978). The quality of seminal plasma (SP) and spermatozoa (SPZ) differs across males with varying capacity for fertilization. The sperm of the most fertile males have higher levels of enzymes related to oxido-reduction activity, respiratory chain, and energy metabolism (Labas et al., 2015). Proteins such as ACE, AvBD10, AvBD9, NEL precursor, and acrosin differ significantly between the most and least fertile males (Labas et al., 2015).

Siegel and Beane (1963) measured the motility, sperm concentration, and semen volume of chickens kept in separate cages and all-male flocks and observed that these traits may differ according to housing conditions and genetic lineages. Chicken sperm viability, mitochondrial activity, and DNA integrity are all impacted by cryopreservation. The freezing-thawing process raises the proportion of dead cells and sperm with fragmented DNA while decreasing sperm viability and

mitochondrial function (Partyka et al., 2010). ROS such as hydrogen peroxide and hydroxyl radicals significantly impair sperm motility, plasma membrane integrity, mitochondrial activity, and DNA integrity, leading to reduced fertility. Oxidative stress leads to lipid peroxidation, which damages sperm membranes. Different chicken breeds exhibit varying semen quality traits, with some breeds showing higher resistance to oxidative stress and better semen quality under stress conditions (Sonseeda et al., 2013). Chicken semen and mammalian semen exhibit significant differences in cryopreservation due to their unique physiological and biochemical characteristics as shown in Table 1.

Table 1 Comparative differences of avian and mammalian semen in terms of physiological and biochemical characteristics.

Parameter	Avian Semen	Mammalian Semen
Cryopreservation success	Lower and highly variable fertility rates post-thaw (2-80%) (Song and Silversides, 2007; Çiftci and Aygün, 2018)	Generally higher and relatively more consistent fertility rates (Çiftci and Aygün, 2018)
Sperm morphology	Unique shape and membrane fluidity (Çiftci and Aygün, 2018)	More uniform morphology across species (Çiftci and Aygün, 2018)
Membrane composition	Higher quantities of polyunsaturated fatty acids, requiring more antioxidant protection (Partyka et al., 2012; Çiftci and Aygün, 2018)	Lower quantities of polyunsaturated fatty acids (Çiftci and Aygün, 2018)
Cryoprotectants	Common cryoprotectants can be toxic or contraceptive (Çiftci and Aygün, 2018)	Common cryoprotectants like glycerol are effective (Çiftci and Aygün, 2018)
Antioxidant enzyme activity	Significant changes in catalase, glutathione peroxidase, and superoxide dismutase activities post-thaw (Partyka et al., 2012)	Less pronounced changes in antioxidant enzyme activities (Partyka et al., 2012)
Lipid peroxidation	Increased lipid peroxidation post thaw (Partyka et al., 2012)	Lower levels of lipid peroxidation post thaw (Partyka et al., 2012)
Sperm viability and motility	Lower post-thaw viability and motility, usually improved with additives like BSA and sucrose (Blesbois et al., 2008; Thananurak et al., 2019; Behnamifar et al., 2021)	Relatively higher post-thaw viability and motility (Thananurak et al., 2019; Behnamifar et al., 2021)
Preconditioning effects	Sublethal oxidative stress (e.g., nitric oxide) can improve post-thaw quality (Feyzi et al., 2018)	Preconditioning less commonly used or needed (Feyzi et al., 2018)
Cryopreservation methods	Use of "pellet" method and dimethylacetamide (Partyka et al., 2012)	Standardized methods with glycerol and other cryoprotectants (Partyka et al., 2012)
Sperm longevity in the female tract	Can survive for several days (Akhtar et al., 2022)	Typically survive for a few hours (Akhtar et al., 2022)

PRINCIPLES OF CHICKEN SEMEN CRYOPRESERVATION

Chicken semen cryopreservation is a critical technique for preserving genetic diversity and ensuring the long-term viability of poultry breeds. The process involves several key steps: collection, dilution, cooling, freezing, and thawing. Each step must be carefully optimized to maintain sperm viability and fertility.

Key Points of Chicken Semen Cryopreservation

Over 70 years of research have led to the current cryopreservation methods for chicken sperm. Nevertheless, no uniform procedure has been created for every breed or strain of chicken. The effectiveness of cryopreservation is affected by numerous important factors, including semen extenders, CPA, pre-freezing modification, semen packaging type, and freezing and thawing rates. Figure 1. illustrates the process of semen cryopreservation, highlighting the key steps involved.

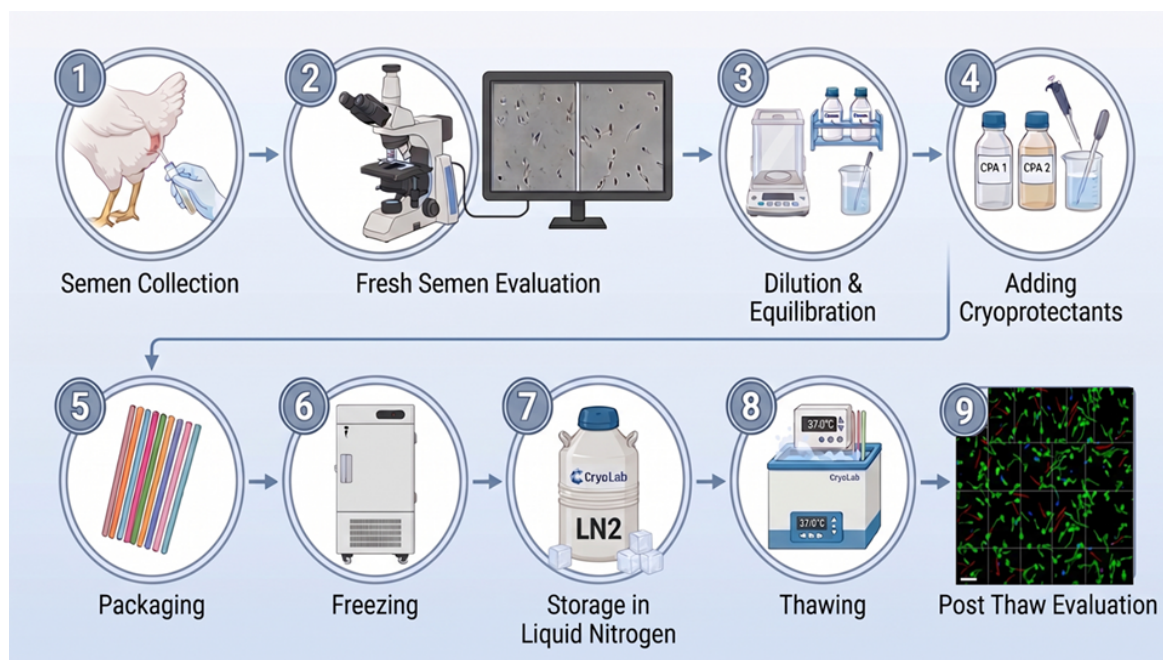


Figure 1 Key steps in the chicken semen cryopreservation process. (1) Semen is collected from the donor animal using appropriate collection techniques. (2) Fresh semen is initially evaluated for quality parameters such as motility, concentration, morphology, and viability. (3) The semen is diluted with a suitable extender and subjected to an equilibration period to stabilize sperm cells under reduced temperature conditions. (4) Cryoprotective agents are added to protect spermatozoa from cold shock and ice crystal formation during freezing. (5) The processed semen is loaded into straws or appropriate packaging units. (6) Controlled freezing is performed, typically using programmable freezers or vapor-phase methods to gradually reduce temperature. (7) Frozen semen samples are stored in liquid nitrogen at -196°C for long-term preservation. (8) When required, samples are thawed under standardized conditions to restore sperm functionality. (9) Post-thaw semen evaluation is conducted using advanced techniques such as computer-assisted sperm analysis (CASA) and fluorescence-based assays to assess motility, viability, membrane integrity, and other functional parameters.

Semen collection

Semen is typically collected from male chickens using non-invasive methods. Two non-invasive techniques for collecting semen from chickens are the cloacal massage and the abdominal massage technique (Burrows and Quinn, 1937; Kucera and Heidinger, 2018). Using abdominal massage method, the bird is restrained while being softly stroked on the back from behind the wings to the tail. The operator can gently squeeze the cloaca to release semen after the bird responds by erecting its phallus. Quails, waterfowls, guinea fowls, turkeys, and chickens can all be prepared using this method (Burrows and Quinn, 1937). Using cloacal massage method, the thumb tip is positioned just below the everted cloaca, and the pad of the index finger is placed on the dorsal side of the base of the tail to stabilize the hand. The handler then repeatedly applies medium pressure while moving the thumb deep and cranially, maintaining the thumb below the cloaca. Several ejaculates can be obtained with this method (Kucera and Heidinger, 2018). The quality and quantity of collected semen can vary based on the breed and individual health of the rooster (Bacon et al., 1986; Lin et al., 2022).

Semen extender

After collection, semen is diluted with an extender to protect sperm during the freezing process. Common extenders include Beltsville Poultry Semen Extender and Lake pre-freezing extender, often supplemented with cryoprotectants like dimethylacetamide (DMA) or dimethylsulfoxide (DMSO) (Sexton, 1980; Th  lie et al., 2019). The Table 2 and Table 3 summarize various extenders and components used in chicken semen cryopreservation, highlighting their applications, findings, and any noted side effects. Valine, melatonin, and BSA have shown positive effects on sperm quality and fertility, while the choice of cryoprotectant and extender can significantly influence outcomes. Optimal concentrations and conditions are crucial to maximize benefits and minimize potential side effects.

Table 2 Summary of Extenders available for avian semen cryopreservation, their applications, findings and their side effects.

Extender	Applications	Findings	Side Effects	References
Thai Native Chicken (TNC) Extender	Thai Native Chicken (TNC) Extender	Higher acrosome integrity and active mitochondria; highest fertility rate among tested extenders.	Lower motility parameters compared to some extenders.	(Thananurak, Chuaychu-Noo, Phasuk, et al., 2020)
Tris-Egg Yolk-Based Extender	Compared with commercial extenders for cryopreservation.	Favorable outcomes for post-thaw sperm quality; suitable for local preparation.	No significant differences in fertility and hatchability.	(Getachew et al., 2023)
Lake Extender	Rooster semen freezing	Lake > Beltsville for motility, viability, fertility; 1 mM cysteamine, 60–80 μ M gamma-oryzanol NPs, and 1% P188 (with 2% glycerol) improve motility, membranes, mitochondria, ROS, fertility/hatchability.	High glycerol is toxic; higher CYS doses (4–8 mM) increase late apoptosis; lower P188 with low glycerol less effective.	(Mehdipour, Kia, et al., 2020; Najafi et al., 2020; Behnamifar et al., 2021; Masoudi et al., 2025;)
Beltsville / BPSE	Freezing rooster semen; chilled storage	Common base for antioxidants (crocin, naringenin, mitoquinol); with these, motility, viability, membranes, fertility and epigenetic status improve.	Unsupplemented Beltsville shows lower motility/fertility vs Lake and higher ROS/apoptosis.	(Askarianzadeh et al., 2018; Mehdipour, Daghigh Kia, et al., 2020)

Freezing procedure

The semen-cryoprotectant mixture is equilibrated at low temperatures (around 5  C) for a specific period. Optimal equilibration times vary, but shorter times (1–30 minutes) are generally preferred to maintain sperm viability (Santiago-Moreno et al., 2011; Zaniboni et al., 2014). The cooling process is critical. Slow cooling rates (1  C/min from +5  C to -20  C) followed by faster rates (30  C/min from -20  C to -80  C) have been shown to maximize sperm viability and fertilizing capacity (Sexton, 1980).

Table 3 Summary of Components (mixed in extenders) available for avian semen cryopreservation, their applications, findings and their side effects

Component	Applications	Findings	Side Effects	References
Valine	Added to semen extender for freezing sperm of endangered chicken breeds.	Improved motility, DNA integrity, and fertility rate in cryopreserved semen.	No specific side effects reported.	(Bernal et al., 2020)
Melatonin	Used as an antioxidant in semen extender.	Enhanced motility, acrosome integrity, and plasma membrane integrity; reduced oxidative stress.	Optimal concentration required to avoid increased ROS and MDA levels.	(Appiah et al., 2019)
Bovine Serum Albumin (BSA)	Supplemented in extenders to improve sperm quality. Increased motility and viability post-thaw; improved sperm motility indicators. No significant side effects reported. Thai Native Chicken (TNC) Extender ⁴ compared with other extenders for freezing chicken semen.	Increased motility and viability post-thaw; improved sperm motility indicators.	No significant side effects reported.	(Behnamifar et al., 2021)
Fetal Bovine Serum (FBS)	Tested at various concentrations for cryoprotection.	Improved motility and reduced oxidative stress at lower concentrations; superior to CLCs.	Higher concentrations resulted in poorer post-thaw quality.	(Blank et al., 2020)
Dimethylacetamide (DMA) and N-methylacetamide (NMA)	Used as cryoprotectants in semen extenders.	DMA showed better in vitro quality; NMA preserved viable embryos better	No significant fertility advantage despite improved sperm quality.	(Mosca et al., 2019)
Sugarcane Extract (SE)	Local extender tested with glycerol and DMSO.	Glycerol showed better semen quality than DMSO.	No significant side effects reported.	(Salifu et al., 2023)
Dimethyl Sulfoxide (DMSO) and Ethylene Glycol (EG)	Used in Ringer's lactate and coconut water-based extenders.	No differences in motility and viability; RLY + DMSO 7% had highest viability.	No significant side effects reported.	(KHAERUDDIN et al., 2020)
Dimethylformamide (DMF) and Ethylene Glycol (EG)10	Used in various diluents for cryopreservation.	EG (8%) and DMF (6%) resulted in acceptable fertility levels.	Thawing temperature critical for success.	(Murugesan and Mahapatra, 2020)

Various freezing protocols exist, including slow freezing in nitrogen vapor and rapid freezing using biological freezer units. For rapid freezing semen is first diluted at room temperature using the semen dilution solution. Second, the semen is allowed to cool for 20 minutes at 4 °C. The temperature of the semen is then lowered to -35 °C at a rate of -7 °C per minute. The temperature of the semen is then lowered to -140 °C at a rate of -20 °C per minute. Ultimately, the semen is placed in -196 °C liquid nitrogen and left there until it is needed (Blesbois et al., 2007; Blanco et al., 2012). Semen is first diluted at room temperature using the semen dilution solution. Second, the semen is allowed to cool for at least half an hour at 4 °C. The temperature of the semen is then lowered to -35 °C at a rate of -1.3 °C per minute. The temperature of the semen is then lowered to -140 °C at a rate of -20 °C per minute. Lastly, the semen is submerged in -196 °C liquid nitrogen and left there until it is needed (Blesbois et al., 2007; Blanco et al., 2012). A medium-rate 2-step freezing method (7°C/min to -35°C, then 60°C/min to -140°C) has been found to yield the best results in terms of sperm motility and acrosome integrity (Santiago-Moreno et al., 2011).

Cryoprotectants

The choice of cryoprotectant is crucial. While glycerol is effective, it can have contraceptive effects. Alternatives like DMA and N-Methylacetamide (MA) are also used, with 6% concentrations generally providing better outcomes than higher concentrations (Zaniboni et al., 2014; Mosca et al., 2020). Glycerol remains the most efficient cryoprotectant for chicken semen but poses contraceptive challenges post-insemination (Lin et al., 2022; Salifu et al., 2023). Alternatives like DMSO, DMA, NMA, and DMF are being explored to mitigate these effects, with varying degrees of success (Choi et al., 2014; Rakha et al., 2018; Mosca et al., 2019; Shanmugam and Mahapatra, 2019; Mosca et al., 2020; Salifu et al., 2023). DMSO and DMF show promise due to their positive impact on post-thaw semen quality and fertility (Choi et al., 2014; Rakha et al., 2018; Mosca et al., 2019; Salifu et al., 2023), while NMA and EG also offer potential benefits under specific conditions (Mosca et al., 2019; Mosca et al., 2020; Murugesan and Mahapatra, 2020). Table 4 provides an evidence-based synthesis of various cryoprotectants, detailing their mechanisms of action, primary advantages, biophysical limitations, and direct impacts on chicken semen cryopreservation success.

Thawing methods

The rate at which frozen semen is thawed significantly impacts sperm quality. Thawing at 5°C for 100 seconds has been shown to be more effective than faster thawing at 38°C for 30 seconds, particularly when using 6% MA as a cryoprotectant (Mosca et al., 2020). Thawing at 37°C for 30 seconds while using glycerol as cryoprotectant showed post thaw motility ranging between 37-71 % under different conditions (Mehdipour et al., 2018; Najafi et al., 2018; Mehdipour et al., 2020b; Yousefi et al., 2021). Thawing at 5 °C for 3 minutes using 5% glycerol as CPA showed post thaw motility about 38 % and fertility about 49 % (Zong et al., 2022), while showed post thaw motility 70% and fertility 77.6% using 6% glycerol (Wu et al., 2020). Thawing at 5°C for 5 minutes showed post thaw motility range between 57.6% to 68.8% and fertility range between 66.8% to 91.9% using 6% DMF as cryoprotectant under different conditions (Chuaychu-Noo et al., 2017; Thananurak et al., 2019; Thananurak et al., 2020a; Thananurak et al., 2020b; Chuaychu-Noo et al., 2021).

Key Mechanisms of Cryoinjury

As with mammalian sperm, chicken sperm is also susceptible to cryoinjury. This cryopreservation-induced damage is primarily attributed to oxidative stress and lipid peroxidation, with effects on the sperm's structural and functional components (Fujihara and Howarth Jr, 1978; Chatterjee and Gagnon, 2001; Guthrie and Welch, 2012; Partyka et al., 2012). Oxidative stress arises from an overproduction of reactive oxygen species (ROS) and disruption of antioxidant enzyme activity (Surai et al., 1998; Brouwers and Gadella, 2003). This imbalance not only undermines the integrity, permeability, and fluidity of the sperm plasma membranes but also affects other structures including the acrosome, mitochondria and DNA (Lamirande and Gagnon, 1993; Guthrie and Welch, 2012). The resulting effect is reduced viability, motility, and overall fertility potential of cryopreserved chicken sperm (Guthrie and Welch, 2012). Figure 2 illustrates the sequential pathway of cryoinjury beginning with thermal and osmotic stressors that induce primary structural and functional damage across vital sperm compartments. This cellular degradation accelerates a cascade of severe oxidative stress and molecular imbalances, ultimately resulting in reduced viability, compromised motility, impaired morphology, diminished in vivo fertility, and execution of apoptotic or necrotic cell death pathways.

Table 4 Common cryoprotectants used in avian semen cryopreservation with their mechanism of action, advantage, limitations, impacts on fertility outcomes and evidence-based conclusion.

Cryoprotectant	Mechanism of Action & Target	Primary In Vitro Advantages	Primary Biophysical Limitations & Toxicity Thresholds	Impact on In Vivo Fertility	Analytical Synthesis & Evidence-Based Conclusion
Glycerol	Extracellular & intracellular osmotic stabilization; stabilizes membrane lipids.	Superior protection of structural and mitochondrial integrity; high post-thaw recovery (>50%).	Threshold:> 11% v/v. Strong contraceptive effect in the avian vagina; requires intensive post-thaw washing/dialysis (Lin et al., 2022; Salifu et al., 2023).	0% fertility if unwashed; up to 50-60% with precise, multi-step dilution washing protocols (Lin et al., 2022).	Exceptional for long-term gene bank preservation of structural material, but limited for direct farm artificial insemination (AI) due to mandatory processing steps.
Dimethyl Sulfoxide (DMSO)	Rapid cell penetration; forms hydrogen bonds with water molecules to prevent intracellular ice.	Avoids vaginal contraceptive effect; maintains DNA integrity and acrosome stability (Wahjuningsih et al., 2026).	Threshold:> 8% v/v. High inherent cytotoxicity at room temperature; increases lipid peroxidation risk if unbuffered (Salifu et al., 2023).	Moderate to High (40-70%); outperforms alternative polyols in specific local native breeds (Rakha et al., 2018).	Highly effective alternative to glycerol for immediate field AI, though requiring optimized antioxidant co-supplementation to control cellular stress.
Dimethylacetamide (DMA)	Amide-based penetration; suppresses ice crystal growth with minimal osmotic pressure shift.	Eliminates post-thaw washing step; allows direct vaginal insemination from straws.	Threshold:> 6% v/v. Highly time- and temperature-dependent cytotoxicity during pre-freezing equilibration (Choi et al., 2014).	Stable moderate fertility (30-50%); however, increasing concentrations fail to improve fertility due to cellular toxicity (Mosca et al., 2019).	Current standard for practical commercial AI workflows due to simplified post-thaw logistics, balanced by narrow processing windows.
N-Methylacetamide (NMA)	Amide-based structural protector; binds heavily to membrane fluid channels.	Stabilizes acrosome integrity; can yield exceptionally higher preservation of subsequent embryo viability (Mosca et al., 2019).	Threshold:> 9% v/v. Negative dose-dependent effect on cell viability; lower in vitro motility retention than DMA (Mosca et al., 2019).	Highly variable (21-90%); can maintain high fertility (> 80%) in specialized rare breeds without washing (Sasaki et al., 2010).	Niche utility; highly effective for specific genetic resource conservation and embryo rescue programs despite modest post-thaw kinetic scores.
Dimethylformamide (DMF)	Small molecular weight amide; rapid penetration kinetics across plasma membrane.	Preserves high mitochondrial functionality and progressive velocity parameters (VCL, LIN) (Mosca et al., 2020).	Threshold:> 6-9% v/v. Induces rapid alteration of the plasma membrane if equilibration exceeds 30 mins (Choi et al., 2014).	Variable (35-60%); relies heavily on specific thawing rate dynamics (5°C vs 38°C) (Shanmugam & Mahapatra, 2019).	A highly promising candidate for maintaining elite progressive kinetic profiles, but requires highly standardized thawing kinetics to avoid membrane collapse.
Ethylene Glycol (EG)	Low molecular weight polyol; rapidly crosses cell membranes to displace water.	Low osmotic shock potential upon initial introduction compared to glycerol.	Threshold:> 8-10% v/v. Lower cryoprotective efficiency in avian cells; associated with high post-thaw DNA fragmentation (Robbaani et al., 2025).	Generally low to poor (< 20%); significantly less effective than glycerol and DMSO in local chicken lines (Murugesan and Mahapatra, 2020; Wahjuningsih et al., 2026).	Suboptimal as a standalone CPA for avian sperm due to membrane vulnerabilities and high DNA damage, but functions well in highly specific combined diluent mixtures.

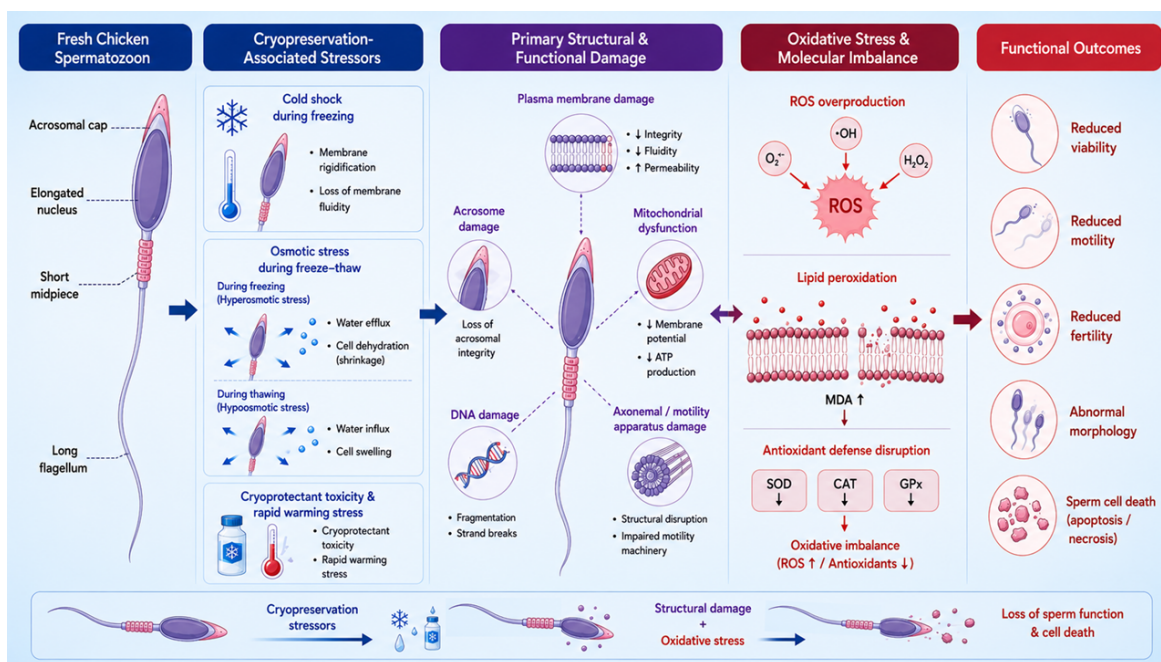


Figure 2 Mechanistic pathway of cryopreservation-associated stressors and their downstream impacts on chicken spermatozoa. This schematic illustrates the sequential biophysical and biochemical cascade from a physiologically intact fresh chicken spermatozoon to compromised functional outcomes caused by the cryopreservation workflow. Characterized by a distinct filiform avian morphology consisting of an apical acrosomal cap, an elongated nucleus, a condensed short midpiece, and a long flagellum, the fresh spermatozoon is subjected to three primary environmental insults during the freeze-thaw cycle: thermal shock inducing membrane rigidification and fluidity loss; osmotic shock driven by phase transitions that cause hyperosmotic cellular dehydration (shrinkage) during freezing and hypoosmotic water influx (swelling) during thawing; and chemical cytotoxicity stemming from cryoprotectant exposure combined with rapid warming stress. These cumulative stressors directly provoke primary structural and functional damage across all vital cellular compartments, resulting in plasma membrane disruption marked by decreased integrity and increased permeability, loss of acrosomal apical cap integrity, genomic DNA fragmentation and strand breaks, axonemal structural disruption that impairs the motility machinery, and mitochondrial dysfunction evidenced by a collapse in mitochondrial membrane potential and subsequent depletion of ATP production. This structural and mitochondrial degradation accelerates the overproduction of intracellular reactive oxygen species (ROS), including superoxide radicals, hydroxyl radicals, and hydrogen peroxide. Excess ROS drives extensive lipid peroxidation across the polyunsaturated fatty acid (PUFA)-rich avian sperm membrane, resulting in an accumulation of malondialdehyde (MDA) byproducts. This oxidative assault concurrently exhausts the endogenous enzymatic antioxidant defense network—signaled by a sharp decline in superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities—culminating in a profound molecular oxidative imbalance. Ultimately, the synergistic integration of these structural injuries and oxidative stress pathways leads to catastrophic functional outcomes, including severely reduced viability, reduced progressive motility, abnormal morphology, and the activation of apoptotic or necrotic cascades, which collectively dictate the post-thaw reduction in in vivo fertility.

Post-thaw viability

The success of the cryopreservation process is ultimately measured by the viability and motility of sperm post-thaw. Optimal protocols ensure high recovery rates of viable and motile sperm, which are crucial for successful fertilization (Sexton, 1980; Zaniboni et al., 2014). The cryopreservation of chicken semen involves meticulous control of each step to ensure high post-thaw sperm viability

and fertility. Key factors include the choice of cryoprotectant, equilibration time, cooling and freezing rates, and thawing conditions. Optimizing these parameters is essential for the effective preservation of poultry genetic resources (Sexton, 1980; Santiago-Moreno et al., 2011; Zaniboni et al., 2014; Mosca et al., 2020).

Oxidative stress and lipid peroxidation

During cryopreservation, increased ROS production and lipid peroxidation (LPO) inflict significant damage on sperm cellular structures (Nguyen et al., 2015; Partyka et al., 2017; Appiah et al., 2019). The resulting oxidative stress impairs sperm motility, activates autophagy and leads to sperm apoptosis and necrosis (Shi et al., 2024). Elevated malondialdehyde (MDA) levels, a marker of lipid peroxidation, further compromise the integrity and functionality of sperm membranes (Partyka et al., 2012; Nguyen et al., 2015; Appiah et al., 2019).

Antioxidant enzyme activity

Cryopreservation also disrupts the activity of key antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD). A decline in CAT activity in the seminal plasma, along with variable responses in GPx and SOD, signals an impaired antioxidant defense system (Partyka et al., 2012; Nguyen et al., 2015; Appiah et al., 2019). Activation of 5'-AMP activated protein kinase (AMPK) offers a potential strategy to restore antioxidant enzyme functions, decrease ROS and LPO, and improve sperm quality post-thaw (Nguyen et al., 2015).

Structural and functional damages

Cryopreservation significantly reduces sperm membrane integrity, acrosome integrity, and mitochondrial activity, leading to decreased sperm viability and motility (Partyka et al., 2012; Partyka et al., 2017; Appiah et al., 2019). Alteration of the carbohydrate components in the sperm membrane glycol conjugates during cryopreservation has been shown to affect sperm immunoprotection and fertility (Peláez et al., 2011). Cryopreservation subjects chicken sperm to intense physical stressors, including cold shock and osmotic imbalance. The transition of water into ice crystals induces severe mechanical and osmotic stress. As water freezes out of the extracellular solution, it creates hyperosmotic conditions, forcing intracellular water to exit and leading to cellular dehydration (Curry and Watson, 1994; Larbi et al., 2024). Conversely, the thawing process creates a hypoosmotic environment as ice crystals melt, causing a rapid influx of water. The unique lipid architecture of avian spermatozoa—characterized by a high concentration of polyunsaturated fatty acids (PUFAs) and a low cholesterol-to-phospholipid ratio, predisposes the membrane to premature phase transitions and stiffening (Ladha, 1998; Blesbois et al., 2005). The freeze-thaw cycle disrupts the equilibrium of membrane lipids, leading to significant cholesterol loss and the disorganization of protein-rich membrane rafts (Cross, 2004; Ushiyama et al., 2016). Cooling linearly reduces membrane fluidity even before visible phase shifts, evidenced by increased fluorescence polarization anisotropy (Giraud et al., 2000; Blesbois et al., 2005). These structural shifts trigger early apoptotic-like changes, such as phosphatidylserine translocation and caspase activation, which impair fertilizing capacity without proceeding to full cellular apoptosis (Martin et al., 2004; Ushiyama et al., 2018).

Impacts of Cryopreservation on Chicken Semen Quality, Freezability and Fertility

The fertilizing ability of frozen-thawed semen was significantly lower compared to fresh semen, with frozen-thawed semen retaining only 1.6% of the

fertilizing ability of fresh semen (Wishart, 1985). Frozen-thawed chicken semen can still achieve reasonable fertilization rates, especially when using optimal cryoprotectants, such as it gave 83.3% fertility with 11% glycerol (Najafi et al., 2018) while it gave 79 % fertility with 6% DMA (Stanishevskaya et al., 2021) and it gave 91.9 % fertility with 6 % DMF (Chuaychu-Noo et al., 2017).

Chicken sperm motility, viability, and membrane integrity play crucial roles in determining its fertility. High motility was found to be associated with better fertility outcomes (Blesbois et al., 2008; Sun et al., 2019). Membrane integrity is also a strong predictor of cryopreservation success as tests for membrane fluidity and integrity correlate positively with fertility outcomes (Blesbois et al., 2008). Most spermatozoa suffered structural damage as a result of the freezing and thawing process, with the mitochondria and mid-piece suffering the most damage (Zong et al., 2022; Zong et al., 2023). Heat shock, osmotic shock, oxidative stress, and ice crystallization damage can all be the cause of these damages (O'Neill et al., 2019).

Compared to some mammalian species, such humans and cows, avian sperm have more polyunsaturated fatty acids in the plasma membrane and less cytoplasm and mitochondria (Najafi et al., 2020). It is thought that these traits increase the cells' susceptibility to freezing-related damage. Water crystallization during the freezing procedure significantly raises the risk of sperm plasma membrane injury (Mehdipour et al., 2020b). According to some studies, oxidative stress which results from an imbalance between cellular antioxidant defense systems and the formation of reactive oxygen species (ROS) during freezing is the primary cause of sperm freezing damage (Peña et al., 2019). When rooster semen is being cryopreserved, ROS can cause harmful alterations (Rui et al., 2017; Ahmed et al., 2021). The plasma membrane and its function may undergo significant alterations as a result of elevated ROS and decreased antioxidants (Simoes et al., 2013). An increase in ROS was also linked to changes in the sperm mitochondrial membrane potential, which changed the activity of enzymes involved in mitochondrial ATP synthesis and increased DNA damage in mitochondria (Nesci et al., 2020; Shahin et al., 2020). Antioxidant systems in sperm and seminal plasma normally eliminate ROS and guard against internal cell damage. Superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), and catalase (CAT) are examples of enzymatic antioxidants (Zong et al., 2023). These adverse effects are mitigated during the cryopreservation of rooster semen by adding antioxidants such vitamin E, selenium, cysteine, superoxide dismutase, L-carnitine, taurine, resveratrol, and hyaluronic acid to the freezing extenders.

Better post-thaw motility, viability, and membrane integrity in chicken semen often do not translate proportionally into fertility gains because fertilization depends on additional steps after insemination, and current in vitro assays capture only part of that biology (Blesbois et al., 2008; Mosca et al., 2019; Bongalhardo, 2025). Conventional post-thaw quality metrics have partial predictive value, but they often overestimate true fertilizing capacity in chickens (Blesbois et al., 2008; Mosca et al., 2019), as fertility depends on oviduct transport, sperm storage tubules, remobilization, and egg interaction, so motility or viability alone is not enough (Bongalhardo, 2025; Masoudi et al., 2025). Chicken sperm must survive insemination, reach and enter sperm storage tubules, remain functional there, reacquire motility, and then bind and penetrate the egg; a semen sample can score well on motility or membrane integrity and still fail at one of these later steps (Bongalhardo, 2025). Embryo outcomes are under-discussed relative to semen quality. One chicken study found better in vitro quality with DMA but no fertility advantage, while NMA better preserved embryo viability, showing that sperm traits linked to fertilization are not identical to those linked to embryo survival (Mosca et al., 2019). Overall, the evidences show that post-thaw motility, viability, and membrane integrity are necessary but insufficient markers of fertility in chicken semen cryopreservation. The field increasingly supports combining conventional assays with fertility trials, sperm-egg interaction tests, and molecular endpoints to

explain why laboratory improvements do not always produce proportional reproductive gains (Mosca et al., 2019; Bongalhardo, 2025; Nonnis et al., 2025).

Impacts of cryopreservation on semen quality and freezability

Freezing and thawing processes significantly impact sperm morphology and DNA integrity in chicken semen. These processes can lead to increased DNA fragmentation, reduced motility, and compromised membrane integrity. Cryopreservation significantly reduces sperm membrane integrity. Because the sperm acrosome, plasma membrane, DNA, and mitochondria may have lost some of their functional integrity during the freezing process, the thawed sperm's viability and survival were decreased (Karakus et al., 2021; Kumar et al., 2021). Compared to several (mammalian) species, poultry spermatozoa have a low level of freezability (Blesbois et al., 2005). The freezing and thawing process caused structural damages to most of spermatozoa with greater damage to mitochondria and mid-piece and mitochondria appeared slightly swollen (Zong et al., 2022; Zong et al., 2023).

Membranes are linked to sperm's resistance to cooling, which is impacted by the extender's chemical makeup or temperature fluctuations. This information can be utilized to forecast the fertility of frozen sperm (Sun et al., 2021). The amount of lipid content on the plasma membrane surface varies throughout avian sperm types, which may impact the sperm's ability to endure varied storage conditions like freezing and cooling (Chuaychu-Noo et al., 2017). Several previous studies have observed a marked increase in membrane damage post-thawing from 2.9% in fresh semen to 66.7% in frozen-thawed semen (Gliozzi et al., 2011; Partyka et al., 2012). There is also a significant reduction in acrosome integrity after cryopreservation (Partyka et al., 2012). Mitochondrial function is also severely affected by the freeze-thaw cycles, leading to decreased ATP production and increased oxidative stress (Partyka et al., 2012; Nguyen et al., 2015). Freezing-thawing processes alter the expression of proteins related to sperm metabolism and structure, affecting the flagellum and overall sperm morphology (Cheng et al., 2015). Spermatozoa's freezability was directly correlated with their fructose, MDA, and SOD levels. Following freezing and thawing, it was shown that SOD activity in the seminal plasma dramatically dropped whereas MDA in the seminal plasma greatly increased (Partyka et al., 2012).

Cryopreservation also leads to a significant increase in DNA fragmentation in chicken sperm from 6.2% in fresh semen to 19.8% in frozen-thawed semen (Gliozzi et al., 2011). This DNA damage is largely attributed to heightened lipid peroxidation and reactive oxygen species (ROS) production during the freezing-thawing process (Partyka et al., 2012; Nguyen et al., 2015). The addition of certain supplements, such as valine, has been shown to mitigate DNA fragmentation (Bernal et al., 2020).

Cryo-survivability differences between individuals and breeds of chicken

The cryo-survivability of chicken sperm varies significantly between individuals and breeds. Rhode Island Red roosters generally show better semen quality and higher fertility rates compared to White Leghorn roosters (Tesfay et al., 2020). The idea that inter-individual variations in sperm freezability are genetically inherited rather than random is explicitly tested and supported by certain findings (Holt, 2000). There is also a significant variation in fertility outcomes among different rooster lines, with some lines showing much higher post-thaw sperm function and fertility rates than others (Long et al., 2010). While no significant differences in fresh semen variables were found between the Birchen and Blue Leonesa breeds, post-thaw sperm viability and DNA fragmentation showed significant variability among different breeders (Bernal et al., 2022). Membrane fluidity, which is highest in chickens and lowest in guinea fowl, also plays a crucial role in sperm freezability

and cryo-survivability. This is in part due to the differences in cholesterol/phospholipid ratio among species (Blesbois et al., 2005). Table 5 highlights the significant differences in cryosurvivability and semen parameters observed among various individuals and breeds of chicken.

Table 5 Cryosurvivability differences in semen parameters between individuals and breeds of chicken.

Parameter	Findings	Reference
Breed variability	No significant differences in fresh semen variables between Birchen and Blue Leonesa breeds	(Bernal et al., 2022)
Breeder variability	Significant differences in post-thawed sperm viability and DNA fragmentation among breeders	(Bernal et al., 2022)
Sperm viability (post-thaw)	Significant differences in post-thawed sperm viability among breeders Breeder D: $58.0 \pm 1.90\%$; Breeder A: $35.2 \pm 7.41\%$; Breeder B: $37.2 \pm 4.09\%$; Breeder C: $22.3 \pm 5.92\%$	(Bernal et al., 2022)
DNA fragmentation (post-thaw)	Significant differences in post-thawed DNA fragmentation among breeders Breeder C: $62.4 \pm 9.91\%$; Breeder B: $31.8 \pm 7.08\%$; Breeder D: $24.5 \pm 5.49\%$	(Bernal et al., 2022)
Membrane fluidity	Highest in chickens, lowest in guinea fowl, intermediate in turkeys	(Blesbois et al., 2005)
Cholesterol/phospholipid ratio	Lower in chickens (0.25), intermediate in turkeys (0.26), higher in guinea fowl (0.29)	(Blesbois et al., 2005)
Cryopreservation impact	Decreased sperm viability and morphological integrity, increased anisotropy in all species	(Blesbois et al., 2005)
Conservation techniques	Efficient cryopreservation of embryonic reproductive cells for genetic diversity preservation.	(Hu et al., 2022)

ADVANCES IN CRYOPRESERVATION TECHNOLOGIES

Recent advancements in chicken semen cryopreservation technologies aim to enhance the efficiency and reliability of genetic diversity preservation. Research efforts have concentrated on optimizing cryoprotectants, improving freezing and thawing protocols, and maintaining sperm quality post-thaw.

Cryoprotectant Formulations

Glycerol and DMA are commonly used cryoprotectants in chicken semen cryopreservation. Studies have shown that the method of adding these cryoprotectants (direct or diluted) does not significantly affect post-thaw sperm characteristics. However, the inclusion of bovine serum albumin (BSA) in extenders with these cryoprotectants can improve sperm motility and viability (Behnamifar et al., 2021). DMA has been found to provide better in vitro sperm quality compared to N-methylacetamide (NMA), although it does not necessarily translate to higher fertility rates in vivo (Mosca et al., 2019). DMSO and ethylene glycol are also used as cryoprotectants. DMSO has been shown to maintain higher sperm viability compared to ethylene glycol in certain extenders (Khaeruddin et al., 2020). However, its effect on embryo viability is less favorable compared to DMA (Mosca et al., 2019).

Innovations in cryoprotectants, such as the use of N-Methylacetamide (Mosca et al., 2019; Mosca et al., 2020), Dimethylacetamide (Mosca et al., 2016; Mosca et al., 2019), and supplements like BSA (Behnamifar et al., 2021) and Sucrose (Mosca et al., 2016; Thananurak et al., 2019), have shown promising results in improving the quality of chicken semen post-thaw. These advancements help reduce toxicity and enhance recovery, contributing to more effective semen cryopreservation protocols. Innovations in cryoprotectants have significantly impacted the quality of chicken semen post-thaw, focusing on reducing toxicity and improving recovery. Below Table 6 is summarizing the effects of various cryoprotectants and methods on chicken semen quality.

Table 6 Current cryoprotectants and extenders (combinations) explored for chicken sperm cryopreservation and their effects on semen quality.

Cryoprotectant	Concentration	Extender	Effects on Semen Quality	Reference
N-Methylacetamide (NMA)	6%	Lake pre-freezing extender with 0.1 M trehalose	Improved sperm membrane integrity, motility, and progressive motility	(Mosca et al., 2019; Mosca et al., 2020)
Dimethylacetamide (DMA)	6%	Lake pre-freezing extender with 0.1 M trehalose	Better in vitro quality and higher viable and progressive motile sperm but no significant fertility advantage	(Mosca et al., 2016; Mosca et al., 2019)
Ethylene glycol (EG)	8%	Kobidil extender	Improved post-thaw motility	(Miranda et al., 2018)
Dimethyl sulfoxide (DMSO)	7%	Ringer's lactate egg yolk (RLY) or coconut water egg yolk (CWY) extenders	Highest sperm viability post-thaw	(Khaeruddin et al., 2020)
Bovine Serum Albumin (BSA)	Supplemented in extenders	Lake and ASG extenders	Improved sperm motility and viability post-thaw	(Behnamifar et al., 2021)
Sucrose	1 mmol	Blumberger Hahnen Sperma Verdünner diluent with 6% DMF	Increased motility, membrane integrity, acrosome integrity, mitochondrial function, and fertility	(Mosca et al., 2016; Thananurak et al., 2019)
Raffinose	1-100 mmol	Blumberger Hahnen Sperma Verdünner diluent with 6% DMF	Negative effects on semen quality and fertility	(Thananurak et al., 2019)
L-carnitine, Hyaluronic acid, sucrose	Various combinations	Ringer acetate-egg yolk (RAEY) diluent	Improved motility, viability, and kinematic variables	(Khaeruddin et al., 2022)

Freezing and Thawing Protocols

Advances in the cryopreservation of chicken semen have focused on optimizing both slow freezing and vitrification techniques. Each method has its own advantages and challenges, and recent research has aimed to determine the most effective protocols for preserving semen viability and fertility. Both slow freezing and vitrification have their respective strengths and weaknesses in the cryopreservation of chicken semen. Slow freezing is more straightforward and cost-effective, while vitrification offers superior preservation of cell integrity and lower DNA fragmentation. Optimizing cooling and warming rates is crucial for maximizing the viability and fertility of cryopreserved semen, with medium-rate two-step freezing and rapid warming being particularly effective strategies.

Slow freezing vs vitrification

Slow freezing typically involves a gradual reduction in temperature, allowing for controlled dehydration and minimal ice formation within cells. This method often uses cryoprotectants like dimethylacetamide (DMA) or dimethyl sulfoxide (DMSO) (Sexton, 1980; Santiago-Moreno et al., 2011). Slow freezing is simpler, less technically demanding, and more cost-effective compared to vitrification. Some studies have shown that this technique maintained higher sperm motility and viability (Sexton, 1980; Mphaphathi et al., 2012). However, slow freezing can result in more unwanted cell clusters and may not be as effective in preserving the integrity of certain cell types, such as primordial germ cells (PGCs) (Tonus et al., 2017).

On the other hand, vitrification involves rapid cooling, transforming the cells and its extracellular milieu into a glass-like state without ice formation. This method requires precise control and often uses higher concentrations of cryoprotectants (Tonus et al., 2017; Patel et al., 2021). Vitrification has been associated with better motility, and lower DNA fragmentation index post-warming. It also tends to preserve the structural integrity of cells more effectively, reducing cryo-injuries (Rienzi et al., 2017; Patel et al., 2021). However, vitrification is expensive, technically more demanding, and requires specialized equipment and expertise to avoid human errors (Tonus et al., 2017; Patel et al., 2021).

Numerous freezing techniques have been studied, including slow and rapid freezing processes with various sperm packing types, and various CPAs (Santiago-Moreno et al., 2017). It is believed that the primary cause of the decreased survival of avian semen using traditional cryopreservation techniques is the freezing rate (Woelders, 2021). According to one study, samples frozen using either a slow controlled freezing method or a rapid freezing method did not affect the sperm's integrity in either liquid nitrogen or vapor-phase nitrogen (Amesse et al., 2003). However, stepwise cooling can be more precisely achieved using an automatic programmable freezer, enabling sperm to progressively adjust to the low temperature to prevent damage (Lin et al., 2022). To prevent the negative effects of CPAs, cell freezing should occur as quickly as possible, yet slowly enough to cause dehydration of the cells (Medeiros et al., 2002). The findings demonstrated that chicken semen may be frozen via fast freezing (Balogun et al., 2020). While the proper cooling rate is chosen based on CPAs and concentrations, it is necessary to explore the cooling rate under various circumstances. DMA is often used with rapid freezing (Chalah et al., 1999), while DMSO and glycerol are typically used with slow freezing (Seigneurin and Blesbois, 1995). The suggested freezing rates for DMF and EG are -15 °C and -1 °C/min, respectively (Tselutin et al., 1999).

Optimization of cooling and warming rates

Current evidence shows that a two-step cooling process of cryopreservation is beneficial. For example, cooling from +5°C to -20°C at 1°C/min, followed by rapid cooling to -80°C at 30°C/min before plunging into liquid nitrogen, yielded optimal results in terms of sperm viability and fertilizing capacity (Sexton, 1980). Medium-rate two-step freezing methods have also been found to yield better post-thaw motility and acrosome integrity compared to slow nitrogen vapor or rapid one-step methods (Santiago-Moreno et al., 2011).

Thawing temperatures also significantly affect sperm survival and motility. Thawing at lower temperatures (e.g., 5°C) has been shown to result in higher survival rates compared to higher temperatures (e.g., 25°C or 41°C) (Mphaphathi et al., 2012). Thawing at 5°C for 4 minutes (Seigneurin and Blesbois, 1995) and at 60°C for 25 seconds (Tselutin et al., 1995) have been included in some studies. The CPAs employed, however, also affect the thawing rate. If semen is frozen with glycerol or DMA, optimal fertility may be achieved by thawing at 5 °C or 60 °C (Chalah et al., 1999). The majority of the summarized investigations demonstrated thawing at 4–5 °C for 3 minutes, 37 °C for 30 seconds, or 60 °C for 6 seconds. For vitrified samples, rapid warming is crucial to prevent ice recrystallization, which can damage the cells. Studies have shown that vitrified PGCs tend to colonize the gonads at higher rates than slow-frozen PGCs, indicating better preservation of functionality (Tonus et al., 2017).

Additives and Supplementation

To mitigate the cryodamage expected during freeze-thaw cycles, various strategies using antioxidants, energy substrates, and proteins have been investigated. These additives and supplements enhance sperm quality by reducing oxidative stress, improving membrane integrity, and maintaining enzyme activity, thereby increasing the overall success of cryopreservation.

The antioxidant melatonin at an optimal concentration of 0.25 mg/mL has shown promise in improving sperm motility, plasma membrane integrity, and acrosome integrity, increasing the activity of endogenous enzymes such as GSH-Px, CAT, and SOD and reducing MDA and ROS levels in cryopreserved chicken semen (Appiah et al., 2019). Sodium alginate, as an energy substrate, has been found to improve sperm longevity, plasma membrane integrity, and the capacity to transit through cervical mucus by enhancing the antioxidant capacity of the semen extender and minimizing phase transitions in sperm membranes (Kumar et al., 2019).

While seminal plasma contributes significantly to cryopreservation by providing protective proteins and enzymes, its elimination and substitution with optimized extenders have been shown in some cases to enhance cryopreservation success. For instance, the removal of seminal plasma and supplementation with bovine seminal plasma (BSP) in the extender has been shown to improve post-thaw sperm motility, viability, plasma membrane integrity, and acrosome integrity, while reducing lipid peroxidation and enzyme release (Dhara et al., 2023). Various additives such as L-carnitine, hypotaurine, taurine, and serine have shown positive effects on the quality of cryopreserved chicken semen, enhancing motility, viability, and reducing oxidative stress. However, some additives like methyl- β -cyclodextrin and tempol were found to be less effective or even detrimental. The choice of cryoprotectant, such as glycerol over DMSO, also plays a significant role in maintaining semen quality. Table 7 summarizes the usefulness and effectiveness of different additives and supplements used for cryopreservation of chicken semen.

Table 7 Additives Used for Cryopreservation of Chicken Semen.

Additive	Description and Effects	Reference
L-Carnitine	Improved sperm motility and reduced DNA fragmentation; increased mitochondrial potential in cryopreserved semen.	(Partyka et al., 2017; Khaeruddin et al., 2022)
Hypotaurine	Resulted in the highest sperm motility and viability post-thaw.	(Partyka et al., 2017)
Taurine	Enhanced sperm viability and reduced lipid peroxidation.	(Partyka et al., 2017)
Bovine Serum Albumin (BSA)	Improved sperm motility and viability in extenders before and after freezing.	(Behnamifar et al., 2021)
Melatonin	Decreased oxidative stress, improved motility, acrosome, and plasma membrane integrity at optimal concentration.	(Appiah et al., 2019)
Cyclodextrins	Methyl- β -cyclodextrin was detrimental, while 2 mg of hydroxypropyl- β -cyclodextrin improved motility and mitochondrial potential.	(Partyka et al., 2018)
Cysteamine, Serine, Ergothioneine,	Serine decreased lipid peroxidation and improved fertility; cysteamine and ergothioneine reduced lipid peroxidation but decreased fertility.	(Thananurak, et al., 2020)
Glycerol	Used as a cryoprotectant, showed higher semen quality compared to DMSO.	(Salifu et al., 2023)
Dimethyl Sulfoxide (DMSO)	Used as a cryoprotectant, but less effective than glycerol in maintaining semen quality.	(Shanmugam and Mahapatra, 2022; Salifu et al., 2023)
Hyaluronic Acid	Improved sperm motility and viability when combined with L-carnitine and sucrose.	(Khaeruddin et al., 2022)
Sucrose	Enhanced fertility when combined with serine in cryopreservation diluents.	(Khaeruddin et al., 2022; Thananurak, et al., 2020)
Tempol	Did not improve post-thaw semen parameters or fertility.	(Shanmugam and Mahapatra, 2022)

CHALLENGES AND LIMITATIONS

Current cryopreservation techniques for chicken semen face significant limitations, including issues with standardization, reproducibility, ethical concerns, and economic considerations. The unique characteristics of chicken sperm and the variability in success rates across different breeds further complicate the development of a universal protocol.

To date, the different protocols developed and tested for freezing chicken semen yield highly variable success rates, making it difficult to standardize a single method effective for all breeds (Çiftci and Aygün, 2018; Thélie et al., 2019; Lin et al., 2022). Common cryoprotectants like glycerol and DMA exert contraceptive or toxic effects, leading to inconsistent fertility outcomes (Çiftci and Aygün, 2018; Lin et al., 2022; Behnamifar et al., 2021). Moreover, chicken sperm cells possess high membrane fluidity and polyunsaturated fatty acid content, making them more susceptible to cryodamage compared to their mammalian counterparts (Gliozzi et al., 2011; Çiftci and Aygün, 2018; Zong et al., 2023). Cryopreservation also can cause significant DNA fragmentation and membrane damage in chicken sperm, further reducing fertility rates (Gliozzi et al., 2011; Zaniboni et al., 2014).

Ethical considerations are important in ensuring welfare-friendly practices, with non-invasive methods like semen cryopreservation being preferred (Nandi et al., 2016; Thélie et al., 2019). Nevertheless, the need for specialized equipment and reagents makes cryopreservation economically challenging for widespread commercial implementation in the poultry industry (Blesbois et al., 2007; Nandi et al., 2016; Çiftci and Aygün, 2018). Moreover, cryopreservation protocols may need to be tailored to different chicken breeds, adding complexity and cost to breeding and conservation programs (Nandi et al., 2016; Zong et al., 2023).

FUTURE PERSPECTIVES: THEORETICAL APPLICATIONS OF EMERGING TECHNOLOGIES

The integration of cutting-edge technologies, such as nanotechnology, microfluidics as well as genetic tools like CRISPR, paves the way for significant strides in the improvement of chicken semen cryopreservation. With the further standardization of protocols and exploration of alternative cryoprotectants, these advancements hold great promise for both commercial applications and the conservation of genetic diversity in poultry.

Nanotechnology Supplementation

Nanoparticle supplementation of semen can mitigate oxidative stress and repair cellular damage during the freeze-thaw processes. Zinc oxide (ZnO), known for their strong antioxidant properties, has been shown to improve post-thaw sperm quality and maintain sperm motility membrane integrity and overall cell viability. This may be explained by their ability to neutralize reactive oxygen species (ROS) and deliver bioactive molecules that support cellular repair mechanisms during the freeze-thaw cycles (Saadeldin et al., 2020). To repair and shield sperm cells from damage, liposomes and exosomes are utilized in the cryopreservation of chicken semen (Saadeldin et al., 2020; Najafi et al., 2020).

During cryopreservation, liposomes—especially those derived from vegetable phospholipids—have demonstrated potential in improving sperm survival and functionality. Antioxidants like vitamin E, which they can contain, enhance sperm motility and viability after thawing. Additionally, liposomes provide hydrophobic compounds with a biocompatible delivery system that increases their bioavailability and protective properties (Mutalik et al., 2014; Shishova et al., 2020;

Taouzin et al., 2022). In a study that was conducted by Najafi et al. (2020), the quality and fertility of post-thawed rooster sperm were investigated in relation to quercetin-loaded Nano-liposomes and nanostructured lipid carriers (NLCs). When compared to control groups, the researchers discovered that adding 15 μ M quercetin to NLCs surprisingly increased sperm motility, viability, membrane integrity, mitochondrial activity, and antioxidant levels. This therapy also improved hatchability and reproductive rates and decreased lipid peroxidation. According to these results, quercetin-loaded NLCs may strengthen quercetin's antioxidant properties, enhancing the fertility and quality of cryopreserved rooster sperm (Najafi et al., 2020). Liposome encapsulated soy lecithin and cholesterol have been proposed as alternatives to traditional chicken egg yolk extenders in semen cryopreservation (Mutalik et al., 2014).

Due to the presence of lipids, antioxidants, and other bioactive substances that regulate and repair spermatozoa, exosomes have the potential to have a pleiotropic effect (Saadeldin et al., 2020). Exosomes, derived from reproductive tract or stem cells, have regenerative capabilities that can repair damaged sperm. They contain bioactive molecules, including antioxidants and lipids, which help regulate and repair spermatozoa during cryopreservation. Exosomes have been shown to improve mitochondrial membrane potential and protect sperm structures, enhancing cryostability (Saadeldin et al., 2020; Kowalczyk and Kordan, 2024).

Microfluidics and Artificial Sperm Reservoirs

Microfluidics and artificial sperm reservoirs are other emerging technologies that have the potential to revolutionize chicken semen cryopreservation. These strategies can facilitate the controlled addition of cryoprotectants and removal of toxic substances, thereby improving the viability and fertility of cryopreserved sperm (Çiftci and Aygün, 2018; Thélie et al., 2019). Although there are still issues with standardizing procedures for reliable fertility results in poultry, but some studies indicate that improvements in cryopreservation techniques, such as microfluidic sperm selection, phosphorus and vitamin B12 supplementation, and optimized cryoprotectant protocols, can enhance sperm motility, genomic integrity, and overall semen quality (Thélie et al., 2019; Yata et al., 2022; Suwimonteerabutr et al., 2023; Saleh et al., 2024).

Scaling Technologies for Commercial and Conservation Use

Scaling cryopreservation technologies for commercial and conservation purposes involves standardizing protocols and improving the efficiency of existing methods. For instance, the use of glycerol as a cryoprotectant has been shown to be effective, but its contraceptive effects necessitate its removal before insemination. Standardizing the removal process can make this method more viable for large-scale use (Thélie et al., 2019; Lin et al., 2022). Additionally, the use of alternative cryoprotectants like N-Methylacetamide (NMA) has shown promise in improving sperm quality post-thaw, which is crucial for both commercial breeding and genetic resource conservation (Mosca et al., 2020; Petričáková et al., 2022).

CONCLUSIONS

In conclusion, this review underscores the pivotal role of advanced cryopreservation techniques in the maintaining the fertility, freezability, and quality of chicken semen for application in genetic conservation and breeding initiatives. Although substantial progress has been made, inevitable challenges such as oxidative stress, lipid peroxidation, and variations in cryo-survivability across breeds and individuals still exist. Promising strategies such as the development of

new cryoprotectants and extender additives, and cutting-edge technologies like nanotechnology, and microfluidics, should be leveraged to enhance cryopreservation outcomes. To ensure these technologies' broader application in both commercial and conservation contexts, future research on protocol standardization and breed-specific adjustments, and ethical considerations will be crucial.

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CONFLICT OF INTEREST

Authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Shahzad Akram: conceptualization (equal); data curation (lead); formal analysis (equal); investigation (lead); visualization (lead); writing – original draft (lead); writing – review and editing (equal).

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Korawan Sringarm: supervision (equal); writing – review and editing (equal).

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Chakorn Khunkaew: writing – review and editing (equal).

Anucha Sathanawongs: conceptualization (equal); project administration (lead); funding acquisition (lead); formal analysis (equal); supervision (equal); writing – review and editing (equal).

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