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Bovine Sperm Cryopreservation: A Review

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ABSTRACT

Bovine semen cryopreservation is a pivotal technique in modern animal husbandry, enabling the efficient preservation and distribution of valuable genetic resources for breeding programs worldwide. Advances in the existing cryopreservation techniques have increased sperm survival and fertility rates, but challenges remain. The efficacy of cryopreservation is measured by post-thaw sperm motility, membrane integrity, and sperm reproductive potential, which are all influenced by parameters such as initial semen quality, type and concentration of the cryoprotectant, cooling rates, and storage duration. The process of cryopreservation causes oxidative stress, osmotic changes, cold shock and cryodamage that alters sperm plasma membrane structure and incurs deleterious changes at the molecular level, leading to compromised sperm integrity. Ongoing research focuses on optimizing procedures, investigating alternate CPAs, and improving cryoprotection with antioxidants and other additions. These enhancements aim to improve the effectiveness of bull sperm cryopreservation, boost genetic diversity and productivity in cattle breeding operations, and facilitate biotechnological applications such as genetic engineering.

Keywords: Bovine, Cryopreservation, Spermatozoa, Cryodamage, Artificial Insemination

Introduction

There is an increased demand to enhance the efficiency and sustainability of animal food production to meet the growing global population (Medeiros et al., 2002). Global beef production in 2023 was estimated at 364 million tonnes, of which 76 million tonnes is contributed by bovine only (Statista, 2023). However, increasing the efficiency of animal product production requires both the development of new technology and an understanding of the reproductive dynamics of the various livestock species (Yánez-Ortiz et al., 2022). One way to increase global food production is to use cryopreserved semen from genetically superior breeds and artificial insemination (AI) to produce high-yield livestock. AI is a key reproductive technology that produces genetically superior breeds to enhance genetic advancement and selection (Bailey et al., 2000). Successful semen cryopreservation enhances the efficiency and success rate of AI, which leads to improvement in the global livestock industry (Bailey et al., 2000).

The process of semen cryopreservation dates back to the 17th century; however, the

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first successful artificial insemination was reported by Spallanzani in dogs in 1784 (Sharafi et al., 2022). A major improvement in the AI industry was witnessed after Polge reported that glycerol can facilitate cell preservation at low temperatures and could be used as a permeating cryoprotectant (Polge et al., 1949). Since the discovery of glycerol as a cryoprotective agent (CPA), significant progress has been made in developing protocols based on a variety of other CPAs for the optimal cryopreservation of bovine sperm (Foote, 2002).

Sperm cryopreservation is crucial for livestock production since it allows for the rapid dissemination of genetic variety and the global dispersion of genetically superior animals; however, this process is very challenging for the sperm and affects its post-thaw viability (Yánez-Ortiz et al., 2022). Sperm encounter physiological and structural obstacles such as osmotic imbalance, oxidative damage, and the development of intracellular ice crystals during cryopreservation (Ugur et al., 2019). These challenges lead to the death of approximately half of the sperm population (Sharafi, 2022), which is very concerning for the AI industry that aims to meet the global need for superior-quality genetics. Sperm cryopreservation treatments may be inefficient due to significant physiological damage suffered by a large number of sperm, resulting in reduced fertility after freezing and thawing. Therefore, new methods are being developed, and cryoprotectants are being explored to improve the survival of sperm during cryopreservation (Nijs et al., 2009).

Cryopreservation of the sperm is still a very challenging task due to the complex physiological nature of the sperm membrane and structure. There have been studies where rapid and slow cooling have been tried, but both methods have certain pros and cons. In both of these methods, cell membrane plays a crucial part, as the water permeability impacts intracellular events and is the primary site of injury (Mazur et al., 1972). Cells can be damaged during cryopreservation by exposure to excessive salt concentrations and the formation of ice crystals internally (Mazur, 1970). Rapid cooling results in ice crystal growth within a cell, which is damaging and potentially hazardous because it can physically disrupt the cell structure and cause it to cease its normal function (Mazur, 1970). Mazur states that reducing the cooling rate can help prevent ice development within the cell (Mazur et al., 1972). Slow cooling, on the other hand, can cause damage from prolonged exposure to high salt concentrations in the solutions surrounding the cell, causing cell dehydration and volume contraction (Ba-Awadh et al., 2023). Sperm cryopreservation is a challenging process because ice crystal formation poses a greater risk than sperm dehydration, affecting the success of sperm freezing. (Ba-Awadh et al., 2023) To reduce intracellular ice crystal formation and sperm dehydration, the optimal freezing process should be gradual and quick. These are the reasons that the cryopreservation of sperm is a challenging task, and there is still a need to optimize these protocols to take sperm cryopreservation to an optimal state (Ba-Awadh et al., 2023).

Methods of cryopreservation

Two traditional freezing techniques employed in sperm cryopreservation are slow freezing and vitrification (Behrman & Sawada, 1966).

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Slow freezing

Slow freezing is time-consuming and can span a duration of 2–4 hours, depending upon the specific species being targeted and necessitates the utilization of either a freezer or a programmable freezer (Silva et al., 2019). Although manual procedures have demonstrated successful sperm freezing, the reproducibility of this procedure may present certain challenges. Due to this reason, many researchers have conducted investigations on programmable freezers (Holt, 2000). The freezers employ a plate as a means of containing the semen straws, which are subsequently cooled by liquid nitrogen contained within a storage tank positioned beneath the plate. The semen straws are cooled gradually at different cooling rates to achieve a chilling process from 20°C to -80°C. Once the freezing is finished, the straws are extracted and stored in liquid nitrogen at -196°C (Santo et al., 2012). Several researchers contend that traditional slow freezing, whether performed manually or automatically, results in significant chemical-physical harm to the sperm, perhaps due to the formation of ice crystals (Santo et al., 2012).

Vitrification

Vitrification is a method that results in the solidification of living cells into a glass-like state without the creation of ice crystals during cooling (Kuleshova & Lopata, 2002). The traditional vitrification approach needs a rapid cooling rate and a high concentration of CPA (Vutyavanich et al., 2010). The conventional vitrification process involves the addition of CPA to the extender media cooling the straws on liquid nitrogen vapours at -80°C and then plunging them directly into liquid nitrogen at -196°C. As the process of vitrification involves the inclusion of a high concentration of CPA, it may lead to metabolic alterations and fatal osmotic damage (Vutyavanich et al., 2010). Vitrification of bovine semen has been documented to produce a significant number of spermatozoa that are non-viable and lack motility (Baiee et al., 2020). The dose of cryoprotectants that cells may tolerate during vitrification has a biological limit, and therefore, the goal of any vitrification strategy is to accelerate temperature change while maintaining the lowest feasible concentration of cryoprotectant (Liebermann et al., 2003).

Cryopreservation damage in sperm

The cryopreservation process incurs significant damage to sperm integrity due to the formation of intracellular ice crystals, oxidative stress, osmotic shock, and other factors. These factors lead to sperm plasma membrane damage, alterations in lipidome and proteome, DNA fragmentation, degradation of mRNA, mitochondrial dysfunction, etc. (Ugur et al., 2019). Cryopreservation also causes organelle damage, leading to spermatozoa with morphologic abnormalities, aberrant acrosomes, altered mitochondria, decreased ATP production, cellular integrity, viability, motility, and fertility (Gillan et al., 2005; Nishizono et al., 2004; Upadhyay et al., 2021).

Efforts have been made to enhance freezing media and protocols, primarily with regard to the addition of cryoprotectants, it is important to remember that the quality

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of frozen-thawed sperm primarily depends on their ability to tolerate temperature changes without losing their primary functions (Sieme et al., 2008).

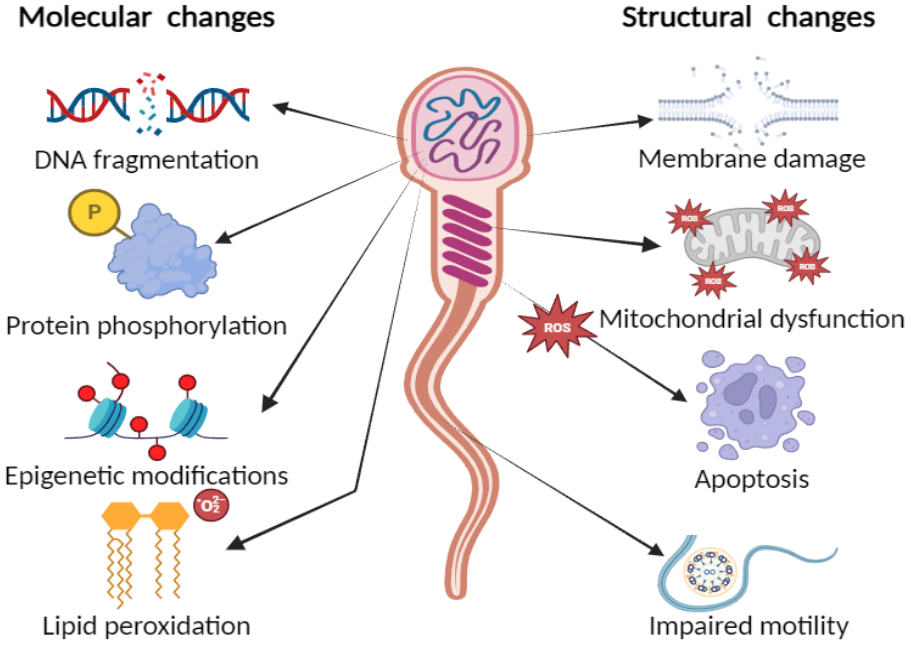

Figure 1: Cryopreservation-induced molecular and structural modifications in sperm

Damage to sperm plasma membrane

The majority of cryodamage in sperm is attributed to the structural integrity of the plasma membrane and is, therefore, connected to the composition of the plasma membrane (De Leeuw et al., 1993). Cryotolerance in bull spermatozoa is influenced by membrane structural characteristics such as cholesterol/phospholipid ratio, fatty acid mapping, hydrocarbon chain saturation, protein/phospholipid ratio, protein composition, and expression levels in seminal plasma (Esmaeili et al., 2015; Parks & Lynch, 1992; Ugur et al., 2019). For instance, certain phospholipids enhance the fluidity of the cell membrane, whereas cholesterol contributes to stability, which appears to enhance the ability of sperm to withstand freezing damage (Sharafi et al., 2022). Membranes having a lower cholesterol-to-phospholipid ratio, along with an asymmetrical distribution of cholesterol, appear to possess greater susceptibility to damage (Holt, 2000). The process of cryopreservation also causes elevated ROS production that result in increased lipid peroxidation disrupting the normal membrane function (Ugur et al., 2019).

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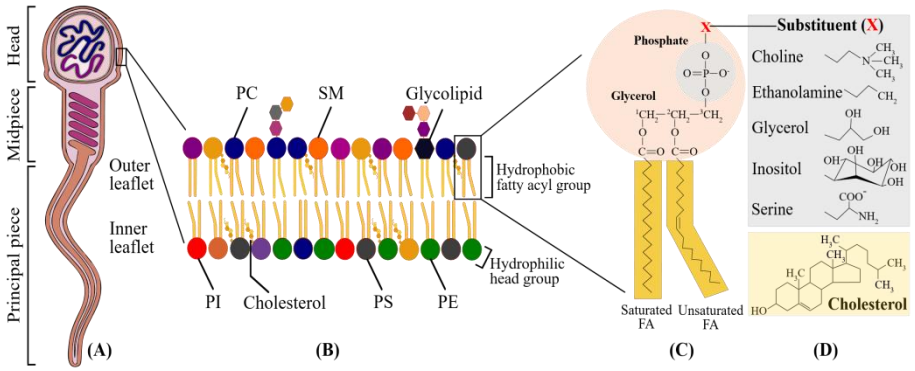

Figure 2: Schematic representation of bovine sperm, sperm plasma membrane, phospholipid molecule and lipid class structures.

Damage at molecular level

Freezing and thawing during cryopreservation harm sperm DNA integrity, making it more susceptible to molecular and epigenetic alterations that influence embryo development (Lewis & Aitken, 2005). This detrimental process has been found to produce chromatin instability, resulting in DNA fragmentation for boar and avian sperm (Fraser & Strzezek, 2007). DNA damage is likely associated with many mechanisms that occur during cryopreservation, including double-strand breaks caused by high amounts of ROS generation (McCarthy et al., 2010), mechanical stress on the genomic regions of DNA molecules, where cell shrinkage causes chromatin compaction to increase (Kopeika et al., 2015).

Epigenetic factors, including protamine, DNA methylation, and histone modifications, play key roles in spermatogenesis are also affected by the process of cryopreservation (Teperek et al., 2016; Ugur et al., 2019). Furthermore, epigenetic factors affect gene expression, which is dynamically regulated during cryopreservation (Zeng et al., 2014). The amount of sperm RNA is easily influenced by freezing-thawing cycles, and some degree of these RNAs is stable (Ugur et al., 2019). Cryodamage can also cause degradation of mRNAs (Giaretta et al., 2017), disrupting protein function and expression levels of fertility-related proteins (Kashir et al., 2011).

To protect the sperm from these harms during cryopreservation, special chemical compounds known as cryoprotectants (CPAs) are added to the semen extender. Other than CPAs, the general recipe of semen extenders includes energy sources, antioxidants, antibiotics, etc. (Ugur et al., 2019).

Cryoprotectants

The crucial aspect for sperm is to retain its functionality in an environment that must offer optimal environment, including optimal pH, energy, temperature, and osmolarity to prevent physical harm. Therefore, cryopreservation media containing CPAs is added to ejaculated semen and helps reduce ice crystal formation and cold shock that cause damage to sperm during cryopreservation (Abdelhafez et al., 2009).

Cryoprotectants are categorized into two types: permeating CPAs and non-permeating CPAs. Permeating CPAs possess high water solubility at low temperatures and have the ability to readily permeate biological membranes, and ideally, exhibit minimum

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toxicity (Wolkers, 2021). Non-permeable CPAs do not penetrate within the cell and thus exhibit their protective effects outside. They are usually bigger and covalently bonded as polymers, dimers, or trimers (Bartolac et al., 2018).

Permeable cryoprotectants:

Permeating cryoprotective agents like glycerol, ethylene glycol and dimethyl sulfoxide (Me_2SO_4) are recognized for their protective effect based on colligative characteristics (Mazur et al., 1972). Permeating cryoprotectants (Holt, 2000) have an amphiphilic nature and tiny size (usually fewer than 100 daltons), enabling them to easily pass through cell membranes and enhance the reorganization of membrane lipids and proteins, increase membrane fluidity, and promote higher dehydration at lower temperatures, thus improving cryo-survival (Holt, 2000). Permeating cryoprotectants interact strongly with water through hydrogen bonding, the freezing point of water decreases, and fewer water molecules are present to form key nucleation sites necessary for crystal formation (Whaley et al., 2021).

Glycerol largely enhances the colligative characteristics of a solution and decreases the salt content upon freezing (Lovelock, 1953). Glycerol molecules permeate lipid bilayers and induce modifications in the diffusion rates of electrolytes across the membrane, including Na^+ and K^+. These changes lead to the osmotic shrinkage of sperm cells, allowing them to withstand low temperatures. Despite the lower permeability of glycerol relative to other cryoprotectants, several effective cryopreservation methods nevertheless incorporate glycerol (Seki et al., 2007). In swine, glycerol and trehalose significantly increased acrosome integrity in comparison to glycerol (Gutiérrez-Pérez et al., 2009). Although glycerol has numerous advantages as a CPA, its toxicity in high concentrations is the subject of some debate. Glycerol in higher quantities is regarded to be damaging in numerous aspects of cell function (Hammerstedt & Graham, 1992). High concentrations of glycerol affect the polymerization and depolymerization of α and β tubulins, the main microtubule proteins in the sperm tail (Alvarenga et al., 2005). Increased glycerol content in thawed pig sperm reduces plasma membrane fluidity (Buhr et al., 2001). An increased concentration of glycerol alters the characteristics of F-actin, a globular protein in the cytoskeleton (García et al., 2012).

One way to lower glycerol toxicity is to substitute glycerol with amides. Amides are a type of cryoprotectant; they cause less harm to sperm during cryopreservation in stallions because of their smaller molecular weight (Alvarenga et al., 2005). Substituting glycerol with dimethylformamide and methyl formamide improved the acrosome integrity, mitochondrial membrane potential, motility, and vitality of horse sperm (Wu et al., 2015). Moreover, a mixture of methyl formamide and glycerol enhanced the integrity and movement of the plasma membrane in cattle sperm (Akhtar et al., 2022).

Among the other major permeating CPAs, ethylene glycol has been used as a substitute for glycerol in many species. Ethylene glycol has shown superior cryoprotectant properties compared to glycerol in bovine spermatozoa (Guthrie et al., 2002). A major advantage of this CPA is that it permeates the sperm membranes more rapidly, leading to decreased hydraulic conductivity and subsequently

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reduced osmotic stress on cells during cooling and freezing (Gilmore et al., 1995; Phelps et al., 1999). Research indicates that ethylene glycol has a less harmful impact on the survival and movement of sperm cells, offering superior protection to the acrosome compared to glycerol (Ball & Vo, 2001). Ethylene glycol has been successfully used as a cryoprotectant in the cryopreservation of dog (Swelum et al., 2011) and horse (Mantovani et al., 2002) spermatozoa.

Dimethyl sulfoxide (Me_2SO_4) is another widely used CPA, which is an amphipathic molecule that dissolves in both aqueous and organic solutions (Santos et al., 2003). Dimethyl sulfoxide was used to freeze the first human oocytes, which led to a live birth after thawing (Chen, 1986). Dimethyl sulfoxide is believed to impact cellular permeability by influencing membrane dynamics in a concentration-dependent manner. Me_2SO_4 at low doses (5%) is believed to reduce membrane thickness, thereby enhancing membrane permeability (He et al., 2012).

4.2 Non-permeable cryoprotectants

In the case of non-permeable CPAs, they are unable to pass through the cell membrane; instead, they cause water to move out of the cell and increase concentrations outside the cell (Sutton, 1992). Commonly utilized non-permeable CPAs include sugars like trehalose, glucose, and galactose (Whaley et al., 2021).

Trehalose is synthesized by a diverse range of species, such as bacteria, fungi, yeast, insects, plants, and certain invertebrates (Elbein et al., 2003). It has been shown to help these organisms survive freezing, among its several roles. The structure of trehalose consists of two glucose units connected by an α-1,1-glycosidic bond. The acetal bond in each monomer avoids the decrease of C-1, enhancing stability at high temperatures and reducing vulnerability to acid hydrolysis in low-pH environments (Whaley et al., 2021). Trehalose as a CPA is expected to affect membrane fluidity by integrating into the phospholipid bilayer, making the membrane more stable during freezing (Aboagla & Terada, 2003). It was reported that trehalose can decrease cryo-capacitation and sustain acrosomal integrity (Reddy et al., 2010). It has also been reported that trehalose reduces acrosomal damage and improves membrane fluidity during cryopreservation, consequently impacting fertility (Ahmad & Aksoy, 2012). Trehalose was found to improve membrane integrity in bulls when added to the semen extender (Hu et al., 2010).

Glucose is another widely used non-permeating CPA that also serves as an energy source for gametes and embryos and is often present in culture media in millimolar quantities (Fuller et al., 2005). In bovine spermatozoa, the low concentration of non-metabolizable glucose analogue, 3-O-methylglucose, improved the post-thaw motility (Bhat et al., 2020). In another study, glucose in the extender media, along with glycerol, improved the sperm quality parameters in bull spermatozoa (Awad, 2011). In boar spermatozoa, extender media containing a relatively higher concentration of glucose resulted in higher post-thaw motility, making glucose a good candidate as a CPA in boar sperm cryopreservation (Reyes et al., 2002).

Table 1: Commonly used CPAs in sperm cryopreservation.

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Permeable CPAs	**Species**	**Reference**
Glycerol	Porcine	(Gutiérrez-Pérez et al., 2009)
Ethylene glycol	Bovine	(Guthrie et al., 2002)
Dimethyl sulfate (Me_2SO_4)	Human	(Chen, 1986)
Dimethyl sulfoxide (DMSO)	Bovine	(Taşdemir et al., 2013)
Formamide	Equine	(Alvarenga et al., 2005)
Acetamide	Porcine	(Yoshino et al., 1993)
Methanol	Equine	(Bass et al., 2004)
Butylene glycol	Bovine	(Suzuki et al., 1993)
Adonitol	Human	(Alvarez & Storey, 1993)
Non-permeable CPAs		
Trehalose	Bovine	(Hu, Zan, et al., 2010)
Glucose	Bovine	(Bhat et al., 2020)
Galactose	Equine	(Swain & Smith, 2010)
Polyethylene glycol	Bovine	(Ohboshi et al., 1997)
Raffinose	Equine	(Swain & Smith, 2010)
Hyaluronan	Porcine	(Peña et al., 2004)
Arabinose	Equine	(Swain & Smith, 2010)
Polyvinylpyrrolidone	Bovine	(Checura & Seidel, 2007)
Lactose	Bovine	(Swain & Smith, 2010)

Among sugars, galactose is also used as a non-permeating cryoprotectant(Swain & Smith, 2010). Galactose has been reported to hinder ice recrystallization during freezing/thawing, showing minimum toxicity at physiological temperatures, and can alleviate osmotic stress (Chaytor et al., 2012).

Extender media

As discussed earlier, sperm faces many challenges, including variable pH (Liu et al., 2016), elevated ROS production (Amin et al., 2018), media toxicity (Ghaniei et al., 2019), osmotic imbalance (Dorado et al., 2019), sperm membrane damage (Layek et al., 2016) etc., during cryopreservation. Researchers have developed a variety of extender media to ensure sperm resilience during chilling and freezing procedures (Baiee et al., 2018). Extenders preserve sperm maintain motility and fertility throughout time by stabilizing the membrane, providing energy substrates, preventing the negative effects of pH and osmolarity fluctuations (Foote & Leonard, 1963). Currently, multiple extenders use a variety of material sources, including animal sources, skimmed milk, egg yolk (Filho et al., 2009), and soybean lecithin (plant

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source) (Layek et al., 2016), which offer different characteristics and issues depending on the type of sperm extender and species (Bustani & Baiee, 2021). Some widely used extender media in bovine sperm cryopreservation are discussed here.

Egg yolkolk-based extenders

Egg yolk is an effective cryoprotectant for sperm cryopreservation at varying doses in different species (Abdel-Khalek, et al., 2018). The exact mechanism by which egg yolk protects sperm during freezing is still unknown (Moussa et al., 2002) however, it is suggested that low-density lipoproteins (LDL) in egg yolk can protect spermatozoa against cold shock and improve sperm motility (Graham & Foote., 1987). Egg yolk is one of the most commonly used extenders for preserving bull sperm because it can protect against membrane damage (Purdy, 2006; Qureshi et al., 2014). Poultry egg yolk can be utilized in sperm extenders; however, some research suggests that other animal egg yolks, such as quail (Bustani & Baiee, 2021), turkey and pigeon (Akhter et al., 2018), and ostrich (Naz et al., 2019), can also be used. Furthermore, researchers discovered that quail egg yolk improved sperm motility and membrane integrity in bull semen better than other bird egg yolks (Akhter et al., 2018). The predominant component of yolk plasma is LDL, followed by livetins (Alexandra, 2013). Livetins are serum proteins composed of albumin, α-2-glycoprotein, and immunoglobulin Y (IgY) (Schade & Chacana, 2007). Egg yolk also contains phosvitin, which is a highly phosphorylated protein with antibacterial and antioxidant effects (Anton et al., 2007). Generally, the percentage of egg yolk in egg yolk-based extenders is 20% (Amirat-Briand et al., 2010; Layek et al., 2016). Tris-buffered egg yolk extenders with fructose and glycerol maintain animal sperm fertility at high extension rates (Bustani & Baiee, 2021).

However, the use of egg yolk comes with a number of drawbacks. As the egg yolk is an animal-derived ingredient, it poses a substantial danger of microbial contamination of the semen, necessitating the use of antibiotics in egg yolk-based semen extenders. Egg yolk contains granular components that threaten the maintenance of the quality of spermatozoa and interfere with a variety of other metabolic processes (Layek et al., 2016). Egg yolk is an incredibly complex substance whose composition varies across batches and changes with diet, and these variables make it impossible to have constant quality and composition in egg yolk (Moreno et al., 2013). Thus, complete egg yolk has some sperm toxicity and interferes with laboratory testing. For these reasons, two significant plant-derived replacements for entire egg yolk have shown potential: soy lecithin and crude soymilk (Singh et al., 2012). In addition, some researchers have shown that egg yolk toxicity can be minimized by lowering the content of granular and high-density material in the media using clarification-repeated centrifugation at low temperatures (Amirat et al., 2005).

Milk-based extenders

Milk has been frequently used to cryopreserve mammalian sperm, usually mixed with arabinose, fructose, or egg yolk (Barbas & Mascarenhas, 2009). Skim milk proteins can buffer semen pH and may also bind heavy metal ions (Perea et al., 2017). Lactose, an important milk ingredient, is hydrophilic and cannot disperse the sperm cell

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membrane, hence protect the cell wall and prevent freezing shock (Namdeo Tukaram et al., 2010; Perea et al., 2017; Rahman et al., 2018). The protective component of milk is most likely micelles of caseins, the main proteins in milk. Caseins (α, β, and κ) are found in milk proteins as heterogeneous colloidal particles called casein micelles (Bergeron et al., 2007). Casein micelles are composed of a hydrophobic core of α and β caseins, surrounded by κ caseins (Dalgleish, 1998). It has been demonstrated that casein micelles extracted from milk can protect bull sperm while stored at 4-5°C (O'shea & Wales, 1966). Furthermore, casein micelles can protect bull sperm during freezing in the presence of glycerol (Bergeron et al., 2007).

Plant-based extenders

Plant-based semen extenders, which are usually produced from soybean lecithin, are easier to modify and are more stable in composition than animal-derived extenders (Mousavi et al., 2023). Soybean lecithin, an alternative to egg yolk, has been created and commercially used for sperm preservation (El-Sisy et al., 2016). Keeping in view the need for biosecurity, plant-based extenders offer an alternative to animal-source extenders, which pose a risk of contamination (Kumar et al., 2015). Therefore, soybean lecithin can be utilized as an alternative to milk- or egg yolk-based extenders for bulls (Layek et al., 2016; Tarig et al., 2017); boars (Pearodwong et al., 2019) and Rams (Forouzanfar et al., 2010) semen cryopreservation. Furthermore, El-Keraby et al. discovered that using a soybean-based extender improves sperm functional motility and lowers bacterial contamination in freeze-thawed bull semen (El-Keraby et al., 2010). Similarly, Zhang et al. demonstrated that a semen extender containing 6% soybean lecithin could improve sperm general and progressive motility, as well as the integrity of the plasma membrane of post-thawed male boar sperm cells (Zhang et al., 2009).

Commercially available extenders

Several commercial extenders are also used to dilute and preserve sperm during cooling and freezing (Bustani & Baiee, 2021). Triladyl® is a commercial egg yolk extender that provides exceptional protection for bull semen against freezing shock (Amirat et al., 2005). INRA 96® is another commercial extender that contains caseinate, which is used to preserve the semen of camel (Malo et al., 2018). BotuSemen® is also an extender using a skim milk base intended to preserve frozen stallion sperm (Hernández-Avilés et al., 2020). Gent® is an egg yolk-based extender that has been used for many years to preserve the semen of donkey (Bottrel et al., 2018). EquiPlus® is a commercial extender containing specific milk proteins intended to preserve the sperm of buffalo (Singh et al., 2012). Bioxcell® is a commercial extender that comprises either milk, egg yolk, or both and is used for cryopreservation of bovine semen (Kaka et al., 2015).

Antibiotics in semen cryopreservation

One of the benefits of AI is to minimize the spread of infections, yet this technology may allow pathogens to be easily propagated through the transmission of contaminated semen (Foote, 2002). Pathogens can harm sperm and lower the quality

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of cryopreserved semen (Diemer et al., 2000). To inhibit the pathogen growth in the cryopreserved semen, antibiotics are added to extender media prior to cryopreservation (Morrell & Wallgren, 2011). Antibiotics such as penicillin, streptomycin, ceftiofur, apramycin, aminoglycosides, lincospectin, tylosin, and gentamycin have been added to semen extenders (Raheja et al., 2018). Antibiotics are also added as a combination in the extender media; for example, the GTLS (gentamycin, tylosin, lincospectin, streptomycin) combination is frequently used in semen extenders (Schulze et al., 2019). Antibiotics, such as penicillins and cephalosporins, are needed in semen diluents because they interfere with the process of bacterial cell wall synthesis, which causes lysis and cell death (Santos & Silva, 2020). Studies show that ciprofloxacin combined with Tris-citric acid diluent is suggested for preserving buffalo semen (Akhter et al., 2013). A mixture of gentamicin-tylosin-lincomycin-spectinomycin has been recommended for diluting the sperm of numerous animals, including bulls and buffaloes (Andrabi et al., 2016). Besides this, other antimicrobial combinations such as ceftiofur/tylosin and ofloxacin have been identified as having a more effective inhibition of bacterial growth in bovine semen (Gloria et al., 2014). Furthermore, the usage of ciprofloxacin added to the Tris-citric acid diluent has been advised for the preservation of buffalo semen (Akhter et al., 2013).

Besides the advantages of using antibiotics, bacterial resistance to antibiotics used in sperm preservation has increased globally in recent years, raising serious concerns. Therefore, researchers are also opting for alternatives to antibiotics, which may include the use of antimicrobial peptides and physical methods.

Alternatives to antibiotics in semen extenders

Antimicrobial peptides

Antimicrobial peptides (AMPs) are a wide range of naturally occurring molecules with strong antimicrobial action. AMPs play an important role in innate immunity because they target microbial pathogens through mechanisms such as membrane disruption, enzyme inhibition, and immunological regulation (Zasloff, 2002). PR-39, a proline-arginine-rich antimicrobial peptide from pig myeloid antimicrobial peptides 36 (PMAP-36) and 37 (PMAP-37), which has been used as an adjuvant to swine semen extenders and was reported to reduce the bacterial growth while enhancing sperm viability (Bussalleu et al., 2017).Antimicrobial peptides are preferred because of their safety, less residue, and low resistance qualities, and their distinct antimicrobial processes demonstrate tremendous potential in fighting antibiotic resistance (Ji et al., 2024).

Physical methods

Physical procedures like centrifugation and filtration can potentially minimize or replace the need for antimicrobials. Many studies have shown the efficacy of physical methods in reducing the propagation of microbes in semen samples. Androcoll™-E is used to remove germs from equine samples, including E. coli in various conditions, proportions in semen aliquots, bacterial counts were reduced by 68%-97% (Morrell et al., 2014). Centrifugation in the Percoll density gradient separates mature spermatozoa

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from diluents, cells, and bacteria (Arias et al., 2017). Separation using density gradients improves the quality of bovine sperm, particularly in cases of excessive viscosity, poor quality, or cryopreserved semen (Lee et al., 2009). In equine semen, colloidal centrifugation with Androcoll™-E before freezing decreased bacterial load and improved post-thaw motility (Guimarães et al., 2015).

Seminal plasma microfiltration is another physical procedure for reducing antimicrobials where the seminal plasma is separated from the sperm by centrifugation, then filtered with a syringe prefilter followed by a syringe filter and added to fertilization medium with or without antibiotic for AI dosages (Barone et al., 2016). This method not only reduces bacterial contamination in pig semen but also improves motility, plasma membrane and acrosome integrity, and mitochondrial activity (Barone et al., 2016).

Antioxidants

Antioxidants are compounds that prevent the production of reactive oxygen species (ROS) and lipid peroxidation (Amidi et al., 2016). During cryopreservation, spermatozoa experience high oxidative stress, which compromises sperm health and viability. Therefore, to cope with increased ROS generation, antioxidants are added to the extender media, which help in maintaining the oxidative stress balance in the sperm. Commonly, enzymatic and nonenzymatic antioxidants are used, and both have proven to be helpful in enhancing cryorecovery and reducing cryoinjury.

Enzymatic antioxidants

Superoxide dismutase (SOD) enzymes in the cytoplasm and mitochondria react with two common ROS molecules, the superoxide anion (O^{-2}) and hydrogen peroxide (Amidi et al., 2016). SODs are metalloenzymes found in all living organisms and are essential components of the antioxidant defence system, actively involved in safeguarding sperm from oxidative stress (Qamar et al., 2023). Multiple findings suggest that adding SOD to semen extender can enhance the freeze-thaw quality of bull (Qamar et al., 2023) and stallion sperm (Cocchia et al., 2011).

Catalase (CAT) is an enzyme located in the peroxisomes that breaks down hydrogen peroxide into water and oxygen to avoid lipid peroxidation of the plasma membrane and is also found in both sperm and seminal plasma in semen (Bauchc et al., 1994). Seminal plasma is the primary source of CAT however developing sperm can exhibit a low level of CAT (Bauchc et al., 1994). Roca et al. found that catalase enhances the fertilizing ability of pig sperm after thawing when used alone or in conjunction with SOD (Roca et al., 2005). Fernandez-Santos et al. showed that catalase prevents DNA damage in bull spem during the oxidative stress of cryopreservation (Fernández-Santos et al., 2009). CAT was also found to enhance freeze-thaw sperm motility, viability, and DNA integrity in the sperm of camel (Malo et al., 2019).

Glutathione peroxidase (GPx) is another key antioxidant enzyme which plays a crucial role in the process of detoxifying lipid peroxide (Tavilani et al., 2008). GPx is found inside sperm and is situated within the mitochondrial matrix (Yeung et al., 1998). There are many studies that show the impact of glutathione on semen cryopreservation in various animals. Silva et al. showed that glutathione at different

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concentrations maintained the acrosome integrity of ram sperm (Silva et al., 2011). Glutathione peroxidase is an important antioxidant that plays a crucial role in safeguarding sperm cells from oxidative damage (Sanocka & Kurpisz, 2004). Glutathione (GSH) is a potent antioxidant that shields bull sperm from free oxygen radicals and enhances motility, plasma membrane integrity, and cell survival

Non-enzymatic antioxidants

Among the non-enzymatic antioxidants, Vitamin C is a naturally occurring water-soluble vitamin with exceptional antioxidant capabilities (Fraga et al., 1991). It shields sperm from oxidative harm by counteracting O_2, H_2O_2, and OH and it also protects sperm DNA from ROS due to its strong antioxidant properties (Fraga et al., 1991). Mangoli et al. reported that vitamin C in humans enhanced sperm motility, vitality, and DNA integrity following vitrification (Mangoli et al., 2018).

Vitamin E is a naturally existing fat-soluble molecule and is mainly present in the plasma membrane of sperm (Qamar et al., 2023). Multiple studies have demonstrated the effectiveness of vitamin E in treating infertile persons with oligoasthenozoospermia caused by OS (Kessopoulou et al., 1995). The study findings indicate that the vitamin E supplementation led to notable enhancements in sperm concentration, motility, viability, and oxidative activity within the seminal plasma of chickens (Eid et al., 2006).

Beta-carotenoids and lycopene are vital elements of the antioxidant defence system, which aid in preserving the structure of plasma membranes, controlling the growth of epithelial cells, and playing a role in spermatogenesis (Hogarth & Griswold, 2010; Qamar et al., 2023). Inadequate consumption of carotenoids can result in decreased sperm motility (Walczak et al., 2013).

Melatonin is another highly effective antioxidant that has practical applications in sperm cryopreservation (Ofosu et al., 2021). Melatonin is used in sperm extenders, which showed promising results in reducing damage from free radicals and oxidative stress in farm animal sperm (Ofosu et al., 2021). Melatonin plays a crucial role in preserving the stability of the plasma membrane by inhibiting the oxidation of membrane lipids caused by free radicals and minimizing the occurrence of leakages across membranes (Reiter, 2000). The antioxidant capacity of melatonin leads to a decrease in cell death caused by oxidative stress by neutralizing O_2^-, hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and nitric oxide (NO), which are highly reactive to the sperm cells (Najafi et al., 2018; Tamura et al., 2020). Studies have shown that the versatile antioxidant melatonin has benefits on sperm cryopreservation in various species, including bovine (Su et al., 2021), ram (Pool et al., 2021), canine (Divar et al., 2022), and human (Minucci & Venditti, 2022).

Table 2: Commonly used antioxidants in sperm cryopreservation.

Antioxidant	Category	Species	Reference
Vitamin C (Ascorbic acid)	Vitamin	Ram	Azawi and Hussein 2013
Vitamin E		Bovine	O'Flaherty et al. 1997
Carotenoids (β-carotene)		Human	Ma et al., 2021

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Selenium	Mineral	Human	Rezaeian et al., 2016
Resveratrol	Herb	Ram	Zhu et al., 2023
Quercetin		Bovine	Avdatek et al., 2018
Rhodiola sacra aqueous extract (RSAE)		Boar	Zhao et al. 2009
Genistein		Ram	Elsayed et al., 2019
Ergothioneine		Ram	Numan Bucak et al., 2014
Rosemary		Bovine	Daghigh-Kia et al. (2014)
Melatonin	Hormone	Bovine	Su et al., 2021
Superoxide dismutase (SOD)	Enzyme	Bovine	Qamar et al., 2023
Catalase (CAT)		Camel	Malo et al., 2019
L-cysteine	Amino acid	Boar	Santos et al., 2024
Glutathione (GSH)	Tripeptide	Ram	Shi et al., 2020

Cryobanking

In the fields of farm animal biotechnologies, cryopreservation emerges as a highly valuable technique for enhancing selection, facilitating the diffusion of genetic advancements, and promoting conservation (Leroy et al., 2011). The current cattle production farming system is at risk due to a loss of genetic variety and disease outbreaks caused by inadequate genetic resources (Sharafi et al., 2022). Cryobanking of gametes and embryos allows for the transmission of genetic information within and between populations, even after the donor has died. This increases the genetic pool of in situ populations, which helps to counteract the loss of genetic diversity and, as a result, decreases the potential for fitness loss due to inbreeding effects (Lermen et al., 2009).

Several gene banks have been established with varied methods and regulations that vary with the breed, species, and country concerned (Burge et al., 2011; Woelders et al., 2006). Different approaches have been proposed to harness genetic resources to optimize the management of genetic diversity in endangered breeds (Sonesson et al., 2002). Cryobanks today store a variety of reproductive cell types, including mammalian sperm and embryos, avian sperm and primordial germ cells, fish sperm and germ cells, and shellfish sperm and larvae (Canovas et al., 2017). Some cryobanks also include somatic cells with the goal that, in the future, these cells will be reprogrammed into ineffective reproductive cells (Canovas et al., 2017).

Depending on the country, numerous methods have been used to sample individuals for national collection. In the Netherlands, the majority of the tested bulls are sampled for preservation in the gene bank (Leroy et al., 2011), while in the USA, the selection of animals for cryopreservation is aimed at optimizing genetic diversity within the collection by sampling animals from clusters determined through computed genealogical relationships (Blackburn, 2009). The National Cryobank of Domestic Animals in France has reproductive cells and somatic tissues from avian, mammalian, fish, and shellfish, preserved as semen, embryos, or larvae, depending on the species (Ref). The advancements in reproductive physiology and biotechnologies have enabled the expansion of the species available in cryobanks (Sharafi et al., 2022).

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CONCLUSION

The process of cryopreservation is an advanced method, but it incurs deleterious effects on sperm quality and fertility. Cryopreservation leads to structural, molecular and plasma membrane changes in the sperm. The processes of cryoinjury in mammalian spermatozoa are of great interest to researchers in the field of cryobiology. There is no certain decision about which method of cryopreservation should be prioritized more, but we can presume that in future, the methods for cryopreservation will be more optimized to get better sperm post-thaw viability and improved fertility rates for cryopreserved sperm. New means of improving post-thaw sperm parameters are applied now, such as adding extenders, antibiotics and antioxidants, to increase the fertilizing capacity of cryopreserved semen.

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