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Review article

Bull sperm cryopreservation: An overview on the current status and future perspectives

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Abstract

Cryopreservation refers to freezing cells or tissues at extremely low temperatures, allowing them to be stored for extended periods while maintaining viability. Cryopreserved bull semen has become an essential tool in cattle breeding programs and commercial cattle production systems. This review provides a detailed analysis of the current methods and challenges in preserving bull sperm using cryopreservation. We explore the effects of cryopreservation on sperm cells, the role of different cryoprotectants, as well as the progress made in the analysis of bull semen. It also highlights the impact of the freezing process on sperm morphology and functionality, emphasizing the importance of optimizing cryopreservation techniques to maintain sperm fertility and viability. The article underscores the significance of cryopreservation technology in cattle genetics and breeding and suggests future research to enhance cryopreservation techniques.

Keywords: Bull, Sperm, Cryopreservation

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Introduction

The cattle industry relies heavily on cryopreservation technology today. Without cryopreservation, it would be challenging to preserve and distribute high-quality bovine genetics from elite bulls. Every time a valuable bull died or became inactive, its genetics would be lost. This would lead to a steady decline and loss of genetic gains in key economic traits made over long-term breeding programs for high milk production, carcass quality, reproductive performance, and health (Moore and Hasler, 2017). Widespread use of cryopreserved semen from top bulls has enabled breeders to achieve great hybrid vigor and maximize productivity. The absence of cryopreservation would restrict the gene pool available for breeding, resulting in higher levels of inbreeding and less productive cattle over time (Menchaca, 2023). Moreover, distributing bull semen to remote or artificial insemination centers would be more challenging and expensive. It would involve maintaining live bulls and physically transporting them for breeding, leading to higher costs and slower dissemination of superior genetics. On the other hand, using live bulls for natural breeding raises the risk of spreading infectious diseases like trichomoniasis. Cryopreservation mitigates this risk and facilitates a safer exchange of genetic material (Nicholas, 1996; Bailey et al., 2003; Lamb et al., 2016).

Over the past few decades, the field of cryopreservation, particularly concerning bull sperm, has seen remarkable advancements. This progress is evidenced by extensive research and innovations in the techniques and understanding of cryopreservation and its impacts on sperm cells. The cryopreservation of bull sperm has evolved significantly, from the initial discovery of the positive effects of simple cryo-protectant agents in the 1940s by Polge et al. (1949) on animal sperm cryoprotection to the development of advanced methodologies that enhance the post-thaw viability and fertility of bull semen. These advancements have greatly influenced cattle production and genetic propagation. However, despite these developments, challenges such as variable post-thaw sperm viability and variations in fertility among breeding bulls still persist, prompting ongoing research in this area (Rodriguez-Martinez, 2012; Ugur et al., 2019). The subsequent sections will delve into the recent understanding of the effects of cryopreservation on bull sperm, the various methods of cryopreservation and evaluation, and potential future directions for improving these techniques.

Effect of cryopreservation on sperm cells

Like all plasma membranes, the sperm cell's plasma membrane is composed of lipids and proteins (Figure 1). Komarek et al. (1964) analyzed the lipid composition of bull sperm and seminal plasma samples separately using thin-layer chromatography and reported that the total lipid content of bovine spermatozoa and seminal plasma accounts for 12.0% and 1.35% of the total dry weight, respectively. The cholesterol content in neutral lipids of sperm and seminal plasma from bulls were 23.3% and 18.8%, respectively (Jain and Anand, 1976). Sperm plasma membrane lipids exist mainly as phospholipids and cholesterol. The phospholipids are organized into a dynamic bilayer, where each molecule consists of a phosphate head and two fatty acyl tails. The ratio of polyunsaturated fatty acids to saturated fatty acids in bull sperm (3.5) is greater than in human (1.0) and ram sperm (2.5) (Poulos et al., 1973).

Cholesterol molecules, with their four hydrophobic carbon rings and a carbon side chain, nestle into the membrane, filling any inconsistencies caused by the varying lengths and saturation levels of the fatty acyl chains (Figure 1). This function of cholesterol stabilizes the membrane structure, particularly at body temperature. Amounts of cholesterol in sperm membranes may determine the cryotolerance of the cell because higher levels of

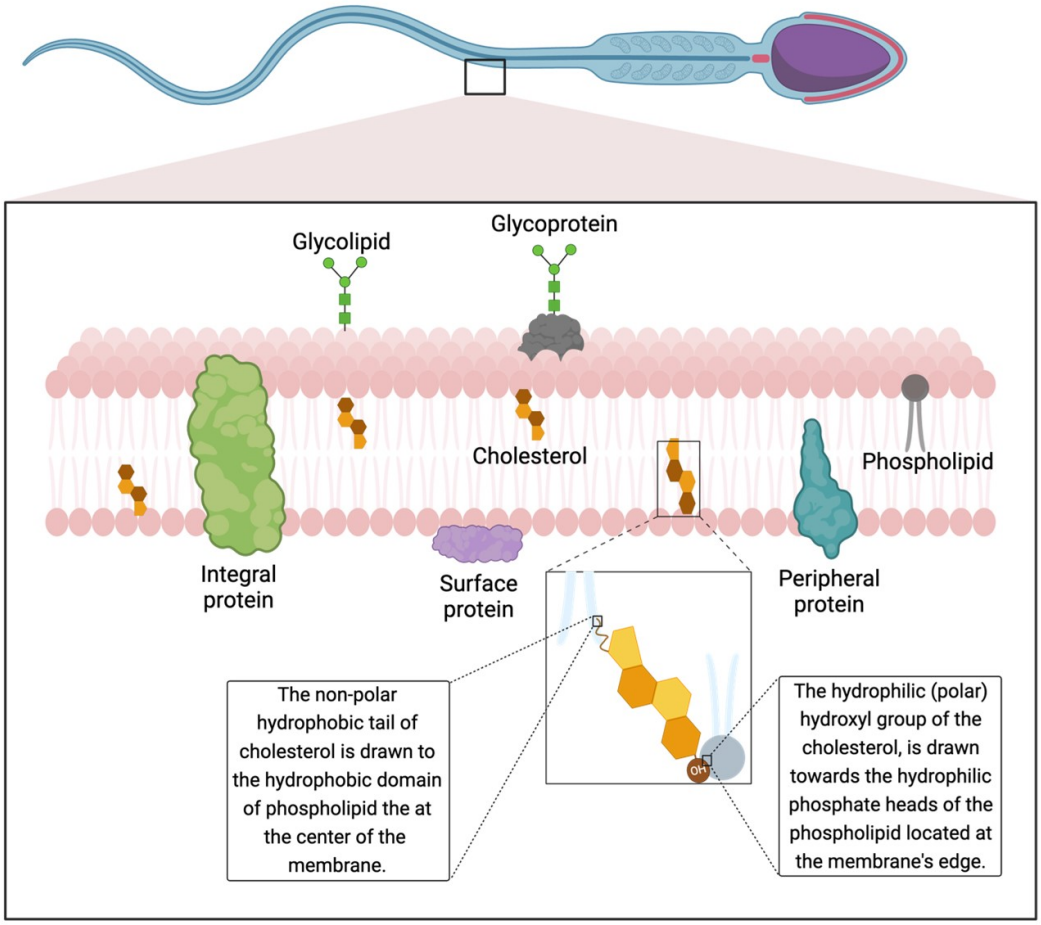

Figure 1: Sperm plasma membrane composition (Created with BioRender.com).

cholesterol result in more rigid and cohesive sperm membranes. Bull sperm contain lower levels of cholesterol compared to human sperm (0.89 μM/10^9 sperm versus 1.438 μM/10^9 sperm, respectively) (Darin-Bennett and White, 1977). Although fresh sperm had slight lipid peroxidation, cryopreserved sperm suffer from higher lipid peroxidation (Bansal and Bilaspuri, 2010; Lone et al., 2016). Ruminant sperm are susceptible to lipid peroxidation due to their high content of polyunsaturated phospholipids (Evans et al., 2021).

It is noteworthy that the cryotolerance of spermatozoa plasma membranes is largely contingent upon the ratio of cholesterol to phospholipids within these membranes. In this regard, various species can be systematically ranked based on their respective plasma membrane cholesterol-to-phospholipid ratios, which, in turn, correlates with their sperm cryotolerance. This ranking is as follows: human sperm exhibits a ratio ranging from 0.99 to 0.83, followed by rabbit at 0.88, bull with a range of 0.40 to 0.45, ram at 0.38, stallion at 0.36, rooster at 0.30, and boar, which has a ratio between 0.20 and 0.26 (Darin-Bennett and White, 1977; Parks and Hammerstedt, 1985; Parks et al., 1987; Mack et al., 1986; Parks and Lynch, 1992).

During cooling, the membrane's lipids transition from a fluid state to a solid, gel-like state, a process known as phase transition (Amann and Pickett, 1987). Unlike a single temperature, this transition occurs across a range of temperatures due to the unique transition points of individual fatty acyl chains. As the temperature decreases, the lipids cluster into "icebergs," causing the proteins to form aggregates within the membrane, which significantly impairs their functionality. However, the presence of cholesterol stabilizes the sperm plasma membrane and retains its fluidity (Figure 2).

As the cooling continues, more lipids solidify, reducing the fluid membrane's proportion until the entire membrane becomes gel-like at the lowest temperature of its transition range. At -5°C, the extracellular solutes, and the cells, along with intracellular water, remain unfrozen but are supercooled. Between -5°C and -15°C, the supercooled intracellular water persists, while extracellular ice begins to form due to a concentration gradient, causing intracellular water to migrate outward and freeze (Gao and Critser, 2000). If the cooling rate is slower than -15°C, most intracellular water will move to the extracellular space, leading to cell dehydration and shrinkage and, ultimately, hyperosmotic shock. Conversely, a rapid cooling rate may prevent dehydration but can cause the formation of intracellular ice crystals (Yeste, 2016). Therefore, the ideal rate for sperm cooling and freezing must be quick enough to prevent dehydration and shrinkage but slow enough to avoid intracellular ice formation (Yeste, 2016). Moreover, the cooling and freezing temperatures can disrupt sperm ATP-dependent ion channels for potassium, sodium, magnesium, and calcium, resulting in depolarization and increased permeability of the plasma membrane and mitochondria. These changes can trigger premature capacitation, cell death, lipid peroxidation in the plasma membrane, and the release of reactive oxygen species (ROS) (Amann and Pickett, 1987). Recently, it has been reported that cryopreservation induces alterations of miRNA and mRNA fragment profiles of bull sperm (Shangguan et al., 2020).

Markers for prediction of bovine sperm fertility and/or freezability

The advancement of the cattle artificial insemination industry heavily relies on the accurate prediction of bull frozen semen fertility. A key determinant of bull-frozen sperm fertility is its resilience to cryogenic damage. Traditional semen analysis methods, involving multiple steps like semen collection, processing, freezing, and fertility trials, are often costly and time-consuming, making them impractical for routine use. Thus, identifying reliable biomarkers for sperm freezability and fertility is of utmost importance. Studying cryopreserved sperm with OMICS tools like proteomics, transcriptomics, and metabolomics provides new

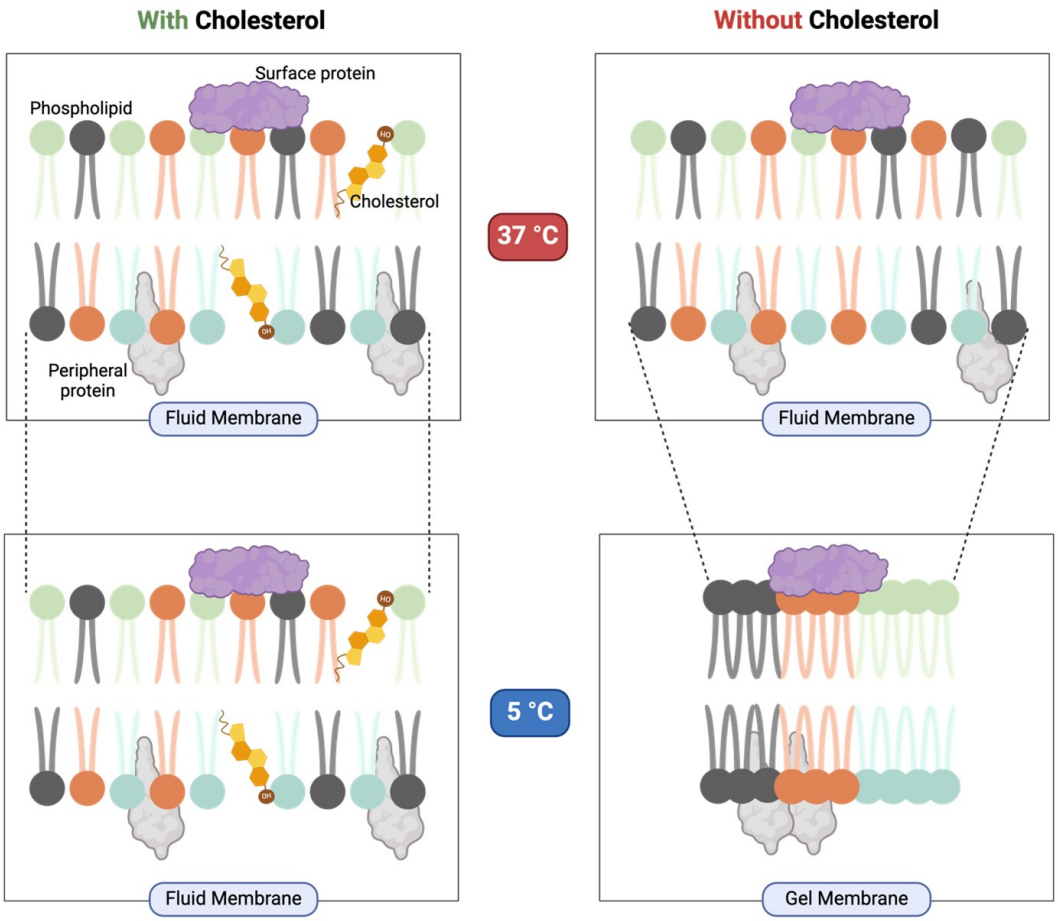

Figure 2: Cholesterol maintains plasma membrane stability during low temperatures (Created with BioRender.com).

insight into tolerance biomarkers and molecular changes from freezing stress. Using systems biology approaches to study cryoinjuries can transform sperm freezing from an empirical method to a highly controlled procedure. This could help optimize cryopreservation methods.

Recent proteomic investigations have focused on identifying specific biomarkers linked to the cryotolerance of bovine sperm. Proteins such as bovine seminal ribonuclease and seminal plasma protein (BSP-5) have been correlated with high semen freezability, while proteins including tubulins, glucose-6-phosphate isomerase, peroxiredoxin-5, spermadhesin-1, gelsolin, sperm equatorial segment protein 1, ATP synthase, calmodulin, glyceraldehyde-3-phosphate dehydrogenase, and secretoglobin family 1D have been associated with low semen freezability (Ryu et al., 2019; Gomes et al., 2020). Other studies have identified positive biomarkers of sperm freezability, including certain proteins (VDAC2, HSP90, AKAP4), RNA transcripts (BCL2L11, CATSPER1), metabolites (amino acids, glycolysis substrates), and gene SNPs (Khan et al., 2021).

In another study, a genome-wide association study was performed to identify SNPs and candidate genes related to various sperm abnormalities (acrosome loss, head/neck/tail defects, motility) in frozen-thawed Holstein bull semen (Dementieva et al., 2024). Significant associations found between specific SNPs/genes and absence of acrosomes (POU6F2, MAP3K7, TCF23, etc.), head anomalies (ORC4, GLRA3, TTK, etc.), swollen acrosomes (LPCAT4, DPYD, bta-mir-137/2420), wrinkled acrosomes (IGFBP3, NPY, MON2, etc.), damaged tails/necks (SAMD5, CLSPN, SLC2A10, etc.), and sperm motility (JPH1, SNCAIP, FSCB, PSMA1). Such Results provide insights into genetic factors and molecular mechanisms affecting the morphology viability of sperm after freezing, laying the foundation for improving cryopreservation protocols and breeding strategies (Dementieva et al., 2024). Using transcriptomics, one miRNA and bta-miR-138 showed significantly lower expression in sperm from subfertile bulls compared to highly fertile bulls. The presence of bta-miR-138 was negatively correlated with sperm oxygen consumption, indicating its potential role in fertility. Three other miRNAs (bta-miR-19b, bta-miR-26a, and bta-miR-7) also showed correlations with sperm function variables (Salas-Huetos et al., 2023).

In summary, the recent advancements in proteomics, transcriptomics, and genomics have significantly improved our understanding of bovine sperm resilience to cryogenic stress. The identification of specific biomarkers, such as proteins, miRNAs, and genetic SNPs, is revolutionizing the selection of high-quality sperm for cryopreservation, thereby enhancing artificial insemination techniques.

Cryoprotectants

The primary aim of a cryopreservation protocol is to ensure the viability of sperm cells, not only throughout the freezing process but also post-thawing. Rehydration, osmotic stress, and plasma membrane disruption are significant challenges sperm cells face during thawing. To optimize sperm cryopreservation effectively, it is crucial to focus on three key aspects: the selection of appropriate cryoprotectants, controlling the cooling and freezing rates, and managing the thawing rate. Cryoprotectants play a vital role in reducing cryoinjury during both freezing and thawing processes. These substances are categorized based on their ability to penetrate the sperm cell. There are permeating and non-permeating types of cryoprotectants (Table 1).

Permeating cryoprotectants, which can infiltrate the sperm cell membrane, alter the cytoplasm's viscosity and reduce intracellular electrolyte concentrations. This action helps in dehydrating the sperm cell during freezing, thereby diminishing the formation of intracellular ice and lessening the degree of osmotic shrinkage (Lovelock and Polge, 1954; Holt, 2000). Glycerol is the most commonly used permeating cryoprotectant in the cryopreservation of mammalian spermatozoa. Other substances like ethylene glycol and dimethylacetamide are also employed as penetrating cryoprotectants (Yeste et al., 2017). However, the penetrative nature of glycerol and similar cryoprotectants can be relatively toxic to sperm cells. The tolerance to

Table 1: Comparison between permeating and non-permeating cryoprotectants (Sathe 2021).

Aspect	Permeating cryoprotectants	Non-permeating cryoprotectants
Penetration	Can penetrate the sperm cell membrane.	Can not penetrate the sperm cell membrane.
Examples	Glycerol, dimethyl sulfoxide (DMSO), ethylene glycol.	Egg yolk, skim milk, sucrose, trehalose.
Mechanism of action	Protect cells by replacing water inside the cell, preventing intracellular ice formation.	Protect cells by creating an osmotic balance, reducing extracellular ice.
Effect on sperm motility	Can potentially affect motility due to intracellular changes.	Less likely to affect motility as they do not penetrate the cell.
Membrane integrity	Can alter membrane properties due to penetration.	Less disruptive to membrane integrity as they do not enter the cell.
Toxicity	Potential toxicity due to intracellular accumulation.	Generally, have lower toxicity as they do not enter the sperm cells.
Concentration	Typically used in lower concentrations.	Often used in higher concentrations to exert effect externally.
Removal after thawing	Require careful removal as they are inside the cells.	Easier to remove as they are not inside the sperm cells.
Effectiveness	Highly effective in preventing intracellular ice formation.	Effective in controlling extracellular ice formation and osmotic stress.

glycerol concentrations varies across animal species due to differences in sperm cell cytoplasm viscosity (Hammerstedt et al., 1978), resulting in varied glycerol tolerances. For instance, semen from bulls and boars can tolerate higher glycerol concentrations than that from stallions (Moore et al., 2006). On the other hand, non-permeating cryoprotectants are those that do not enter the sperm cell membrane. They function extracellularly by stabilizing the plasma membrane and lowering the extracellular compartment's freezing point, consequently reducing the formation of ice crystals around the sperm cells (Hammerstedt et al., 1990). This category includes sugars such as lactose, trehalose, dextran, and proteins (Hezavehei et al., 2018). Animal-derived proteins, like chicken egg yolk and fat-free skimmed milk, have been widely used in the cryopreservation of sperm from various animals. Nevertheless, there is a growing interest in utilizing animal-free cryoprotectants to eliminate the risk of viral or other disease transmissions.

Various extenders and additives (Table 2) have been utilized to mitigate the cold shock impact on bovine sperm, aiming to improve sperm quality and fertility rates post-thawing.

Commonly, chicken egg yolk is incorporated into bovine semen extenders at a concentration of 20%. The cryoprotective efficacy of egg yolk is largely attributed to the presence of low-density lipoproteins (LDL), which coat the sperm plasma membrane and preserve its components during the freeze-thaw cycle (Medeiros et al., 2002; Bergeron et al., 2004). It has been suggested that proteins in seminal plasma when bound to sperm, lead to destabilization of the cell wall. This destabilization is caused by the efflux of cholesterol and phospholipids, rendering the sperm more vulnerable to cold shock during freezing (Manjunath et al., 1994; Thérien et al., 1998). LDLs play a role in binding to seminal plasma proteins and reinforcing the sperm plasma membrane, thereby increasing sperm cryotolerance during freezing. As an alternative to egg yolk, milk-based cryoprotectants have been employed for bovine sperm cryopreservation. The protective effect of these cryoprotectants is mainly due to casein (Bergeron and Manjunath, 2006). Casein interacts with seminal plasma proteins, preventing the loss of lipids from the sperm plasma membrane (Bergeron and Manjunath, 2006).

The concerns regarding the use of animal-based products in semen extenders have risen, primarily due to risks of bacterial or xenobiotic contamination, presence of endotoxins, interference with sperm evaluation, variability in egg yolk composition, and potential compromise to the integrity of sperm cells (Layek et al., 2016). Consequently, the development of animal protein-free media has been initiated (Aires et al., 2003). These concerns have spurred interest in seeking alternatives to animal-based semen cryoprotectants for the artificial insemination industry.

Plant-based cryoprotectants have emerged as an alternative to milk and egg yolk-based extenders. For instance, soybean lecithin has been used as a plant-based extender, providing adequate protection for sperm during cryopreservation while reducing the risk of disease transmission. Comparisons between animal protein-based extenders (e.g., Triladyl, BullXcel, Laciphos) and animal protein-free extenders (e.g., OptiXcell, AndroMed, Biociphos plus) have been made. While *in-vitro* sperm parameters showed some differences, the pregnancy rate and the 56-day or 60-day non-return rates were not significantly different (van Wagtendonk-de Leeuw et al., 2000; Aires et al., 2003; Muiño et al., 2007; Murphy et al., 2013).

In the context of bull sperm freezing using protein-free extenders, it has been observed that the plasma membrane of sperm cells loses cholesterol during freezing (Bailey et al., 2000). Adding cholesterol to the freezing medium has enhanced post-thawing sperm parameters (Purdy and Graham, 2004; Ma et al., 2006). Recently, bull sperm was successfully cryopreserved without the use of any animal or plant protein (Anzar et al., 2019). This was achieved by treating bull semen with a cholesterol-cyclodextrin complex (CC), followed by dilution in an extender containing glycerol and tris salts (TG). The post-thaw sperm parameters were comparable to those frozen in exogenous protein-based extenders, and the protein profile of fresh sperm was similar to that of sperm frozen in CC+TG, suggesting the potential of CC+TG as a promising extender (Anzar et al., 2019).

In bull sperm cryopreservation, the incorporation of nanoparticles and nanovesicles (like liposomes and exosomes) has been explored to assess their impact in reducing cryoinjury. Nanoparticles, derived from natural herbs or metals, function by reducing oxidative stress, decreasing cell apoptosis, and enhancing plasma membrane integrity. Liposomes, which are synthetic nanovesicles made of a spherical single lipid bilayer, can be produced through sonication of lipid suspension (sonicated liposome) (Graham and Foote, 1987) or by extrusion, passing the lipid suspension through extruders with nano-sized pores (extruded liposome) (Röpke et al., 2011). These liposomes integrate into the sperm plasma membrane, helping to repair damage caused by freezing and thawing. Exosomes, which are nano-sized extracellular vesicles released from cells, can decrease sperm cryoinjury by reducing ROS and lipid peroxidation during the freeze-thaw process, delivering antioxidant enzymes, miRNA, and mRNA (Jahanbin et al., 2015; Khalil et al., 2019; Mousavi et al., 2019; Franchi et al., 2020; Saadeldin et al., 2020).

Another method of bull sperm cryopreservation is sperm encapsulation. This process involves creating a mixture of sperm suspension and sodium alginate solution, which is then combined with certain ions (such as barium ions) to form alginate gel beads (Nebel et al., 1985). These beads are subsequently loaded into semen straws, which are then subjected to freezing. This freezing can be executed using programmable freezers or through traditional methods such as immersing in liquid nitrogen vapor using Styrofoam boxes. Encapsulation of sperm offers enhanced protection during cryopreservation. Moreover, it facilitates a con-

Table 2: Different types of cryoprotectants that have been used for bull sperm cryopreservation.

Cryoprotectant	Composition/nature	Beneficial effect	Concentration	Reference
Penetrating cryoprotectants				
Glycerol	A simple polyol compound	Reduces ice crystal formation, stabilizes cell membranes	2-12%	Rasul et al. (2007); Papa et al. (2015)
DMSO (dimethyl sulfoxide)	An organosulfur compound with a high polar nature	Penetrates cell membranes and reduces ice crystal formation	2-6%	Snedeker and Gaunya (1970); El-Harairy et al. (2011)
Ethylene glycol	Low molecular weight, an organic compound used as an antifreeze replacement for glycerol	Lowers the freezing point, reduces osmotic stress	3-7%	Forero-Gonzalez et al. (2012); Saberivand et al. (2023)
Propylene glycol	A synthetic organic compound similar to ethylene glycol	Reduces ice crystal formation and membrane damage	12%	Seshoka et al. (2016)
Methanol	Low molecular weight Simple alcohol, a replacement for glycerol	Lowers freezing point and reduces ice formation	1%	Awad (2011)
Sorbitol	Sugar alcohol	Has a cryoprotective effect and reduces cell damage	1 g/L	Verberckmoes et al. (2004)
Non-penetrating cryoprotectants				
Sodium citrate	A salt of citric acid	Acts as a buffer and has a mild cryoprotective effect	1.5-2.9%	Cragle et al. (1955); Pileckas et al. (2014)
Trehalose	A disaccharide sugar composed of two glucose molecules	Stabilizes sperm membranes and protects against dehydration	25-200 mM	Hu et al. (2010); Öztürk et al. (2017)
Sucrose	A disaccharide sugar composed of glucose and fructose	Acts as an osmoprotectant and prevents cellular dehydration	0.1-0.5 M	Chen et al. (1993); Woelders et al. (1997)
Lactose	A disaccharide sugar found in milk	Helps in osmotic balance and protects against cellular dehydration	11.5%	Pileckas et al. (2014)
Raffinose	A trisaccharide sugar composed of galactose, glucose, and fructose	Cryoprotective and antioxidant properties	25 mM	Tuncer et al. (2011)
Polyethylene glycol (PEG)	Synthetic polymer, less toxic than DMSO	Enhances sperm survival, reduces ice formation	5%	Abavisani et al. (2013)
Plant-based proteins and extracts				
Soybean lecithin	Phospholipid complex from soybeans	Replaces egg yolk, reduces the risk of disease transmission	1-5%	Phillips and Spitzer (1946); Aires et al. (2003); Layek et al. (2016)
Aloe vera extract	Extract from *Aloe vera* plant	Enhances membrane integrity, antioxidative properties	0.25-1%	Boonkong et al. (2019); Singh et al. (2020)
Green tea extract (catechin)	Extract from green tea leaves-Polyphenolic compound	Acts as an antioxidant and protects sperm cells from damage	1.5, 5, 10, 25, and 50 μg/mL	Înanç et al. (2019); Susilowati et al. (2021)
Quercetin	Flavonoid compound	Acts as an antioxidant and enhances sperm motility	25, 50, 100 and 200 μg/mL	Tvrdá et al. (2016); Avdatek et al. (2018)
Curcumin	Bioactive compound from turmeric	Acts as an antioxidant and protects sperm DNA	0.5 and 2 mM	Bucak et al. (2012); Salman et al. (2021)
Animal-based proteins				
Egg yolk	Emulsion of lipids and proteins from eggs	Protects spermatozoa during freezing and thawing	5-20%	Thun et al. (2002); Amirat et al. (2004)
Bovine serum albumin (BSA)	Protein from bovine blood serum	Stabilizes sperm membrane, provides antioxidant protection	0.5-6%	De Leeuw et al. (1993); Ashrafi et al. (2013)
Casein and sodium caseinate	Protein derived from milk	Improves sperm motility and viability	2%	Diniz et al. (2020)
Minerals				
Zinc (nano form)	Metallic element	Improves sperm motility and antioxidant activity	1-10 mM	Jahanbin et al. (2021)
Selenium (nano form)	Metallic element	Protects spermatozoa from oxidative damage	0.5-1.5 μg/ml	Khalil et al. (2019)
Magnesium	Metallic element	Enhances sperm motility and viability	0.5-1 mM	Eidan et al. (2015)
Vitamins				
Vitamin E	Fat-soluble vitamin	Protects spermatozoa from oxidative stress	2 mg/mL	Hu et al. (2011b)
Vitamin C (ascorbic acid)	Water-soluble vitamin	Acts as an antioxidant that improves sperm quality	2.5 mM	Eidan (2016)
Vitamin B12	Water-soluble vitamin	Enhances sperm motility	2.5 mg/mL	Hu et al. (2011a)
Antioxidants and other compounds				
Melatonin	Hormone	Acts as an antioxidant and protects against oxidative damage	0.25– 0.1 mM	ChaithraShree et al. (2020)
Honey	Natural substance	Acts as an antioxidant and osmoprotectant	2.5%	Yimer et al. (2015)
Antifreeze proteins	Protein	Prevents ice recrystallization	0.1, 1, 10 and 100 μg/mL	Prathalingam et al. (2006)

Table 3: Different probes and stains used for flow cytometry analysis of bovine sperm.

Stain	Category	How it works	Reference
Peanut agglutinin (PNA)	Acrosome integrity	Binds to acrosomal contents of the sperm, assessing acrosome reaction and integrity.	Cross and Watson (1994)
Concanavalin A (Con A)	Acrosome status	Binds to specific sugar residues on the acrosome, used to evaluate acrosome integrity and reaction.	Jankovičová et al. (2008)
FITC-Pisum sativum agglutinin (FITC-PSA)	Acrosome status	Binds specifically to the outer acrosomal membrane, used to assess acrosome integrity and status.	Jankovičová et al. (2008)
Alexa fluor phalloidin	Actin filament visualization	Binds to F-actin in sperm, allowing for the visualization and study of cytoskeletal structures.	Rajamanickam et al. (2017)
Annexin V	Apoptosis	Binds to phosphatidylserine, which translocates to the outer leaflet of the plasma membrane during apoptosis.	Dogan et al. (2013)
Oregon Green 488 BAPTA-1 AM	Calcium binding	A calcium indicator is used for measuring changes in intracellular calcium concentrations.	Qin (2008)
Fura-2	Calcium concentration	A fluorescent dye is used for measuring intracellular calcium levels, which is important for sperm motility and capacitation processes.	Dragileva et al. (1999)
Fluo-4 AM	Calcium Ion concentration	A fluorescent dye is used for detecting changes in intracellular calcium levels, which is important for sperm motility and function.	Bucher et al. (2019)
Nonyl acridine orange	Cardiolipin	Binds to cardiolipin, a component of mitochondrial membranes, useful in assessing mitochondrial health.	Uğuz et al. (2014)
CellTracker Green CMFDA	Cellular function	Stains live cells, allowing assessment of cellular function and viability over time.	Puglisi et al. (2010)
Bodipy FL C5-ceramide	Ceramide content	Used for labeling ceramides, offering insights into cell membrane composition and health.	Moreno et al. (2000)
Ethidium homodimer-1, homodimer-2 and bromide	Dead cells/ DNA damage	Penetrates cells with damaged membranes, used for identifying dead or damaged sperm cells.	García-Herreros and Leal (2014)
SYTOX (blue, gold, green, and orange)	Dead cells (Viability)	Penetrates only cells with compromised membranes. Have a high affinity for nucleic acid. Useful for distinguishing between viable and non-viable sperm cells.	Moya et al. (2022)
DRAQ5	DNA	Deep red fluorescent dye that binds to DNA allows for detailed analysis of nuclear morphology and DNA content.	Lamy et al. (2017)
SYBR Green I	DNA	Binds to DNA, providing a more sensitive alternative to other DNA dyes for assessing DNA content and integrity.	García-Herreros and Leal (2014)
Acridine orange (different concentrations)	DNA and RNA	At different concentrations, it can be used to differentiate between DNA and RNA, providing insights into nucleic acid content.	Andraszek et al. (2014)
Hoechst 34580	DNA content	Similar to Hoechst 33342 and 33258, it's used for staining DNA to assess the content and integrity of sperm nuclei.	Kumar et al. (2017)
Hoechst 33258	DNA content and Integrity	Binds to DNA, allowing for the assessment of DNA content and integrity in sperm nuclei.	Kumar et al. (2017)
Hoechst 33342	Viability and DNA integrity	Stains live spermatozoa and allow for assessment of DNA integrity and sperm viability.	Hallap et al. (2006); Duran and Hufana-Duran (2017)
TUNEL assay (terminal deoxynucleotidyl transferase dUTP nick end labeling)	DNA fragmentation	Detects fragmented DNA, a marker of apoptosis or severe DNA damage in sperm.	Takeda et al. (2015)
Acridine orange	DNA integrity and damage	Fluorescent dye is used to assess DNA integrity and damage in sperm.	Andraszek et al. (2014)
PI/YOYO-1 combination	DNA integrity and viability	YOYO-1 is a potent nucleic acid stain used with PI to evaluate both DNA integrity and cell viability.	Duran and Hufana-Duran (2017)
Chromomycin A3	DNA packaging	Binds to G-C-rich regions of DNA, used to assess DNA packaging and integrity in sperm.	Simões et al. (2009)
Bromodeoxyuridine (BrdU)	DNA synthesis	Used to measure DNA synthesis, indicating spermatogenesis activity and DNA replication.	Anzar et al. (2002)
Rhodamine-phalloidin	F-actin	Specifically binds to F-actin, useful for studying sperm cytoskeleton and morphological integrity.	Flaherty et al. (1986)
Calcein blue	General cell viability	A fluorescent dye is used for assessing general cell viability, giving a quick assessment of sperm health.	Bucher et al. (2019)
Calcein violet 450 AM	General cell viability	A cell-permeant dye that emits violet fluorescence when hydrolyzed is used for assessing general cell viability.	Bucher et al. (2019)
BCECF AM (2',7'-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, ecetoxymethyl Ester)	Intracellular pH	A fluorescent dye used to measure intracellular pH is important for understanding sperm physiology.	Vredenburgh-Wilberg and Parrish (1995)

Table 3: Continued

Stain	Category	How it works	Reference
SNARF-5F AM	Intracellular pH	A fluorescent dye used to measure intracellular pH is important in sperm physiology and capacitation processes.	Chávez et al. (2019)
BODIPY 581/591 C11	Lipid peroxidation	Sensitive to changes in lipid peroxidation, indicative of oxidative stress and membrane damage in sperm.	Brouwers and Gadella (2003)
LysoSensor green DND-189	Lysosomal activity	A fluorescent dye that accumulates in lysosomes, allowing for the assessment of lysosomal activity in sperm cells.	Jones et al. (2013)
LysoTracker	Lysosomal activity	Fluorescent dye is used for labeling and tracking lysosomal activity within cells, indicating cellular health and functionality.	Thomas et al. (1997)
Merocyanine 540	Membrane fluidity	Assesses the fluidity and stability of the sperm plasma membrane, indicative of sperm functionality.	Hallap et al. (2006)
M540 Bodipy	Membrane fluidity and integrity	Used to assess sperm membrane fluidity and integrity, crucial for sperm functionality.	Bernecic et al. (2019)
Carboxyfluorescein diacetate	Membrane integrity	Measures membrane integrity, as it can only enter cells with compromised membranes.	Garner et al. (1986)
PKH26	Membrane labeling	A fluorescent cell linker for long-term membrane labeling, useful for tracking sperm in studies of fertilization and motility.	Pagano et al. (2020)
DiBAC4(3) (Bis-(1,3-dibutyl barbituric acid) trimethylene Oxonol)	Membrane potential	A voltage-sensitive dye used to assess changes in membrane potential is important for sperm function.	Thundathil et al. (2006)
DiOC2(3) (3,3'-diethyloxacarbocyanine iodide)	Membrane potential	A lipophilic dye is used for assessing membrane potential relevant to sperm function and vitality.	Nascimento (2008)
DiOC6(3) (3,3'-dihexyloxacarbocyanine iodide)	Membrane potential	Used for assessing membrane potential, crucial for sperm function and viability.	Varela et al. (2020)
Potentiometric membrane dyes (e.g., TMRE, TMRM, JC-1)	Membrane potential	A group of dyes used for assessing mitochondrial membrane potential, crucial for mitochondrial health and function in sperm.	Garner and Thomas (1999); Treulen et al. (2018); Maulana and Kaiin (2023)
MitoTracker	Mitochondrial activity	Selectively stains mitochondria in live cells and is used to assess mitochondrial activity and health.	Garner et al. (1997)
Rhodamine 123	Mitochondrial function/ mitochondrial membrane potential	Stains active mitochondria, used to assess mitochondrial functionality and health.	Celeghini et al. (2007)
MitoSOX red	Mitochondrial reactive oxygen species (ROS)	Selectively target mitochondria to measure the production of reactive oxygen species, indicating oxidative stress.	Blanco-Prieto et al. (2023)
Quantum dots	Multiple targets (depending on conjugation)	Nanocrystals that can be conjugated with various molecules to study different aspects of sperm biology.	Sutovsky and Kennedy (2013)
DAF-FM diacetate diaminofluorescein diacetate	Nitric oxide (NO) production	Used for detecting nitric oxide production in cells, providing insights into cellular signaling processes in sperm.	Martínez-Pastor et al. (2010)
Aniline blue	Nuclear maturation	Stains immature sperm nuclei, indicating issues in nuclear maturation.	Alfadel et al. (2023)
DAPI (4',6-diamidino-2-phenylindole)	Nucleic acid staining	Binds strongly to DNA, used for staining the nuclei and assessing DNA content and integrity.	Komsky-Elbaz and Roth (2018)
LDS-751	Nucleic acids	A fluorescent dye that binds to nucleic acid is useful for evaluating sperm cell viability and nucleic acid content.	Botta et al. (2019)
SYTO (green or red)	Nucleic acids	A green-fluorescent nucleic acid stain that is used for assessing nucleic acid content and integrity in sperm.	Thomas et al. (1997); Birck et al. (2010)
TO-PRO-3 iodide	Nucleic acids	A DNA stain that is useful for discriminating between live and dead sperm cells based on nucleic acid integrity.	Grundler et al. (2004)
DCFDA (2',7'-dichlorofluorescein diacetate)	Oxidative stress	Measures reactive oxygen species (ROS) production, indicating oxidative stress levels in sperm.	Okano et al. (2019)
SNARF-1	pH indicator	Used to measure intracellular pH, an important parameter in sperm maturation and function.	Ballester et al. (2007)
Fast green FCF	Protein content	Stains protein-rich structures within sperm, useful in assessing protein content and distribution.	Way et al. (1995)
Fluorescein isothiocyanate (FITC)	Protein localization and function	Used to label proteins and assess their localization and function within sperm cells.	Thomas et al. (1997)
DCFH-DA (2',7'-dichlorofluorescein diacetate)	ROS	A cell-permeable probe that is oxidized by ROS, forming a fluorescent compound, is useful for studying oxidative stress.	Gürler et al. (2016)
DHR (dihydrorhodamine)	ROS	Converts to rhodamine 123 in the presence of ROS, useful for assessing oxidative stress in sperm.	Gürler et al. (2016)

Table 3: Continued

Stain	Category	How it works	Reference
Hydroethidine (dihydroethidium)	ROS	Oxidized by ROS to ethidium, which binds to DNA, allowing for the assessment of oxidative stress in sperm cells.	Mostek et al. (2017)
H2DCFDA (2',7'-dichlorodihydrofluorescein diacetate)	ROS detection	Measures the production of ROS, providing insights into oxidative stress levels in sperm.	Murphy et al. (2013)
Monobromobimane	Thiol groups	Reacts with thiol groups, particularly glutathione, indicative of cellular redox state and health.	Salman et al. (2023)
ThiolTracker violet	Thiol groups	A fluorescent probe that specifically labels thiol groups, indicating cellular redox state and protein status.	Rocha-Frigoni et al. (2016)
7-Aminoactinomycin D (7-AAD)	Viability	Identifies non-viable cells by intercalating into double-stranded DNA.	Varela et al. (2020)
Propidium iodide (PI)	Viability	Penetrates only dead or membrane-compromised sperm, indicating non-viable sperm.	Garner et al. (1994)
SYBR-14	Viability	Combines with PI for live/dead distinction. Stains live sperm nuclei bright green.	Garner et al. (1994)
YO-PRO-1	Viability and membrane Integrity	Penetrates only the membranes of apoptotic or dead cells, used in conjunction with PI for viability assessment.	Hallap et al. (2006)
FluoZin-3 AM	Zinc ion concentration	A zinc-sensitive dye is used for detecting intracellular zinc levels, which are important in sperm metabolism and function.	Zoca et al. (2023)

trolled release of sperm from the alginate gel beads, potentially leading to improved conception rates. Studies have shown that pregnancy rates achieved with encapsulated cryopreserved sperm are comparable to those obtained with conventionally cryopreserved sperm (Nebel et al., 1993; Perteghella et al., 2017). The technique of encapsulating bovine spermatozoa in alginate shows promise in enhancing the release and survival of sperm within the uterus, thereby prolonging its viability. Nonetheless, further refinement and optimization of this technology are required for its practical application in the field.

Choosing bovine sperm before freezing is a more effective strategy than selecting bulls based on their ability to withstand freezing. This approach minimizes the risk of inadvertently favoring unwanted traits in the gene pool. Methods for collecting competent bovine sperm include sperm migration (such as swimming-up), sperm filtration (like using Sephadex beads), and colloid centrifugation (for example, single layer centrifugation) (Januskauskas et al., 2005; Morrell and Rodriguez-Martinez, 2011; Salman et al., 2023). A newer technique involves using rheotaxis and thermotaxis (Nagata et al., 2019). This process involves incubating the sperm in a fluid that rotates slowly (rheotaxis) and has a temperature gradient from 25°C at the bottom to 30°C at the top (thermotaxis). The motile, viable sperm migrate to the warmer upper layers, where they can be collected. This method has shown significant improvements in sperm quality post-thaw compared to controls (Nagata et al., 2019). Another study explores the potential of a centrifuge-free commercial device called MIGLIS® for selecting high-quality frozen-thawed bovine sperm. The MIGLIS method shows promise in improving sperm quality (motility, viability, and acrosome integrity rates) and reducing ROS concentrations compared to conventional centrifugation-based techniques. Moreover, the blastocyst formation rates were similar, while the intracellular ROS concentrations of embryos fertilized with spermatozoa were selected using the MIGLIS method compared with conventional centrifugation-based techniques (Nguyen et al., 2024).

Current status and advancement in bull semen analysis

The field of bovine andrology has faced challenges in accurately analyzing sperm motion and morphology. Traditional methods like bright-field and differential interference contrast (DIC) microscopy are subjective, lack precision, and are limited in providing detailed, quantitative data about spermatozoa. The need for an objective, automated system to analyze sperm effectively was paramount for advancements in reproductive medicine, research, and clinical practice.

The solution to this problem was the development and refinement of computer-assisted sperm analysis (CASA) systems. These systems represent a significant technological advancement over traditional sperm analysis methods, offering automated, precise, and objective data. Early CASA systems required significant manual input and were less efficient. Modern systems integrate advanced imaging technologies and software for more detailed and automated analysis. While CASA systems provide more accurate and precise data than manual analysis, they have limitations in predicting male fertility due to the complex nature of sperm attributes and fertilization processes (Amann and Katz, 2004). For example, CASA does not adequately consider the complex flagellar waveforms of spermatozoa, hindering the understanding of cell motility. Therefore, high-fidelity computer-assisted beat-pattern analysis (CABA) was introduced as a statistical approach to distinguish between samples based on complex flagellar beating patterns (Walker et al., 2020).

Flow cytometry is widely used in bull sperm analysis for assessing sperm integrity and functionality. Benchtop flow cytometers and versatile markers allow for measuring various sperm parameters, from viability to reactivity to external stimuli. Flow cytometry aids in sorting sperm for potential fertilization and determining chromosomal sex. It has applications in sperm freezing, sperm selection, and sperm sorting. Routine spermiogram evaluations are suitable for identifying infertility but not for predicting fertility levels (Hossain et al., 2011). Flow cytometry provides objective and repeatable analyses of sperm, even with small sample sizes (DeJarnette et al., 2022). Flow cytometry is used to enumerate the total sperm count per ejaculate or straw accurately.

Different fluorescent probes like SYBR-14/propidium iodide (PI) are used to assess viability and membrane integrity. Flow cytometry can also evaluate membrane permeability, stability, early changes, and acrosome integrity using various probes (Table 3). However, Labeling cells for evaluation can alter the shape and size of the spermatozoa. Moreover, stepwise regression models indicated that including multiple semen quality attributes like CASA at 0 and 3 h, flow cytometry viability, and DNA Integrity did not significantly increase the predictive power of semen quality (DeJarnette et al., 2022). The inclusion of multiple semen quality attributes explained only about 3% of the total variance in sire conception rate (SCR) fertility deviations, which means a limited correlation between semen quality and fertility was found (DeJarnette et al., 2022). Therefore, a multidisciplinary collaboration like digital holography, super-resolution microscopy, and next-generation sequencing (NGS), artificial intelligence (AI) is crucial for further progress in improving sperm and genetics analysis and improved access to point-of-care assays (Dai et al., 2021). These new promising techniques for sperm evaluation can be adopted for more accurate bull sperm analysis.

The study of bull sperm cells' morphology is traditionally conducted using optical microscopy. Traditional optical microscopy uses X- and Y-plan; therefore, it is not enough to provide a detailed analysis of the sperm cells. Obtaining a com-

prehensive view necessitates a meticulous z-axis scanning of the biological specimen to gather various focal planes, which are subsequently processed to construct a three-dimensional (3D) representation of the subject being examined. Atomic force microscopy (AFM) offers more in-depth morphological details. AFM operates by scanning a surface with a fine-tipped probe to map out the surface's topography at a nanometric scale, thereby providing a highly detailed morphological image (Allen et al., 1996; Saeki et al., 2005; Carvalho et al., 2013). However, despite its precision, AFM is not widely adopted in the animal production industry. This is primarily due to the intricate preparation required for the samples and the significant costs associated with AFM equipment, making it less feasible for widespread use (Ferrara et al., 2015).

Holography emerges as a cutting-edge method for non-invasive, quantitative examination of cells and tissues, eliminating the need for staining or labeling. Specifically, digital holography (DH) has proven effective in analyzing the morphology of bovine sperm cells. A notable aspect of DH is its ability to generate a 3D image from a single captured hologram, bypassing any mechanical scanning (Di Caprio et al., 2010). This technique significantly enhances the ability to handle quantitative data and perform various numerical analyses. Such capabilities are crucial in exploring the relationship between atypical sperm morphology and male infertility (Ferrara et al., 2015).

A full label-free analysis of bovine sperm cells using a novel experimental setup that integrates DH microscopy and Raman spectroscopy (RS) (Ferrara et al., 2015; De Angelis et al., 2017). DH microscopy provided high-resolution images and quantitative 3D reconstructions of sperm heads, identifying morphological irregularities, including a notable "protuberance" in the post-acrosomal region. Raman imaging further confirmed this anomaly, attributing it to protein vibrations, possibly related to centrioles in the region connecting the sperm tail and head. Additionally, this setup distinguished between X and Y-chromosome-bearing sperm cells, although physical parameters like head size and volume were not definitive indicators. RS's non-invasive detection of DNA content and plasma-membrane proteins proved more effective, achieving over 90% accuracy in identifying the sperm chromosome (Ferrara et al., 2015).

A partially spatially coherent digital holographic microscope (PSC-DHM) was developed to provide quantitative phase imaging (QPI) and distinguish normal vs stressed sperm cells based on nanoscale morphology changes. Sperm cell motility and morphology under bright field microscopy are currently the main criteria used to evaluate sperm. However, factors such as oxidative stress, cryotolerance, and heat can negatively affect the quality of sperm cells and their potential to fertilize by altering subcellular structures that are not visible under bright field microscopy. Therefore, the PSC-DHM system was developed to distinguish differences between normal sperm cells and cells under stressed conditions. Phase maps were reconstructed for a total of 10,163 sperm cells acquired from the PSC-DHM system (2,400 control cells, 2,750 cryopreserved cells, 2,515 oxidative stressed cells, and 2,498 ethanol-affected cells). Seven deep neural networks (DNNs) were employed to classify the phase maps into normal vs stressed sperm cell categories. When validated on the test dataset, the DNNs provided an average sensitivity of 85.5%, specificity of 94.7%, and accuracy of 85.6%. The QPI + DNN framework demonstrates the potential for improving diagnostic efficiency in semen analysis regarding fertilization potential (Butola et al., 2020).

Another recent technique for sperm analysis is stochastic optical reconstruction microscopy (N-STORM). N-STORM is a super-resolution microscopy technique that can achieve spatial resolutions of up to 10 nanometers. This level of resolution allows for the visualization of subcellular structures, such as centrioles, in much finer detail. N-STORM allows researchers to capture the position of both the sperm head and tail at the microscale and centriolar substructure details at the nanoscale. N-STORM enables the analysis of individual sperm cells, providing insights into the dynamic movement of structures within the sperm neck during tail-beating cycles. N-STORM has been crucial in identifying and studying atypical centrioles and the process of centriole remodeling during spermatogenesis (Royfman et al., 2024).

High-resolution 4-D imaging of sperm was developed recently for imaging of freely swimming human sperm cells without the need for staining. The problem addressed is the limitations of current imaging techniques in assessing the 3-D morphology and dynamics of sperm cells, which impacts both biological assays and clinical use. The method captures the 3-D morphology of the sperm head, including internal organelles, and the dynamic motion of the flagellum (Dardikman-Yoffe et al., 2020).

Conclusion

Despite the advancements in traditional semen analysis methods, they still show limited predictive power for semen quality and fertility. Therefore, multidisciplinary approaches integrating advanced techniques like digital holography, super-resolution microscopy, and artificial intelligence are crucial for progress in sperm and genetics analysis. Innovative methods like digital holography (DH) and Raman spectroscopy (RS), partially spatially coherent digital holographic microscope (PSC-DHM), and stochastic optical reconstruction microscopy (N-STORM) have shown potential in providing more accurate and comprehensive sperm evaluation. The adoption of these advanced techniques for a more detailed analysis of bull sperm is vital for understanding fertility and improving cryopreservation methods.

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