New approaches for long-term conservation of rooster spermatozoa

Martina Janosikova,*[,1] Kristyna Petricakova,* Martin Ptacek,* Filipp Georgijevic Savvulidi ,* Jana Rychtarova,† and Josef Fulka Jr.†

*Department of Animal Sciences, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, 165 00 Praha, Suchdol, Czech Republic; and †Department of Biology of Reproduction, Institute of Animal Science, 104 00 Praha, Uhříněves, Czech Republic

ABSTRACT In contrast to the livestock industry, sperm cryopreservation has not yet been successfully established in the poultry industry. This is because poultry sperm cells have a unique shape and membrane fluidity, differing from those of livestock sperm. The objective of this review is to discuss the cellular and molecular characteristics of rooster spermatozoa as a cause for their generally low freezability. Furthermore, here, we discuss novel developments in the field of semen extenders, cryoprotectants, and freezing processes, all with the purpose of increasing the potential of rooster sperm cryopreservation. Currently, it is very important to improve cryopreservation of rooster sperm on a global scale for the protection of gene resources due to the incidence of epidemics such as avian influenza.

Key words: cryopreservation, poultry, cryoprotectants, oxidative stress

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INTRODUCTION

Methods for long-term preservation of sex cells are continuously being developed to preserve genetic diversity and to increase gene flow across populations. The success of cryopreservation is determined by many criteria and conditions of a physical and chemical nature. These conditions must guarantee the preservation of the structures of individual cell organs and membranes during the freeze−thaw procedure and allow for the maintenance of their function (Mohammad et al, 2021). The process of cryopreservation and insemination is routinely applied in cattle. For that reason, our approaches are discussed and compared with dairy- or meat-purpose cattle.

Freezing rooster spermatozoa is the only option for sex cell conservation due to the impossibility of freezing yolk-laden eggs (Long, 2006). Rooster cryopreserved spermatozoa are associated with extremely variable fertility (Lake, 1986; Hammerstedt and Graham, 1992; Buss, 1993; Long et al., 2010; Thélie et al., 2019). Low tolerance to the cryopreservation procedure of poultry sperm can be explained by the cellular and molecular characteristics of poultry spermatozoa (Figure 1); for example, poultry sperm membranes contain higher quantities of polyunsaturated fatty acids than mammalian sperm and, therefore, may require higher antioxidant protection. Commonly used cryoprotectants for cryopreservation have a contraceptive or even toxic effect due to the peculiarity of poultry sperm cells (Çiftci and Aygün, 2018).

Interspecies or even interbloodline differences in spermatozoa tolerance to cryopreservation determine the fertility of cryopreserved semen. It is essential that the development of successful freezing procedures involves more than the identification or application of novel cryoprotectants and additives (Holt, 2000). Although the first major development in poultry sperm cryopreservation was performed in the 1930s−40s (the discovery of glycerol's cryoprotective properties), its toxicity to avian cells remains an unsolved task for workers and scientists in the poultry industry (Long, 2006). Greater fertility for cryopreserved rooster spermatozoa is needed. If improved cryopreservation techniques are developed, they can be used to efficiently preserve these valuable poultry genetics. At the same time, it will be possible to share these genetics globally by sending cryopreserved sperm.

Different Reproduction Biology

Spermatozoa are highly differentiated haploid cells with highly condensed chromatin inside the cells and

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[1]Corresponding author: janosikova@af.czu.cz

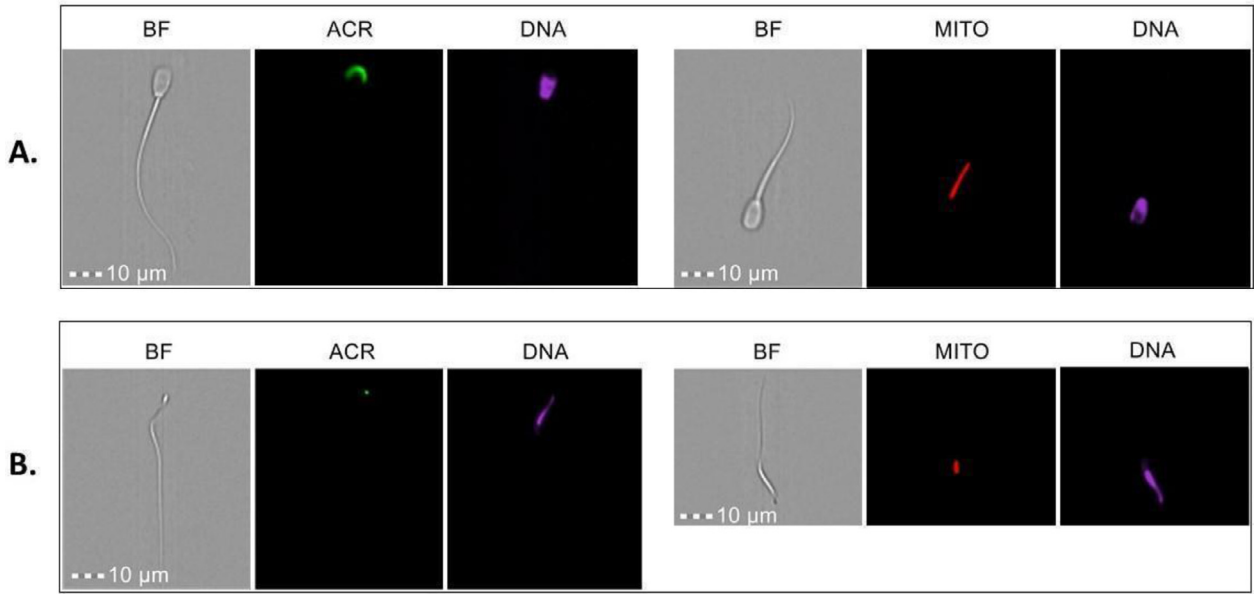

Figure 1. Morphological differences between mammalian and avian spermatozoa (A - bull; B - rooster). Spermatozoa stained with PNA-lectin/FITC (acrosome, ACR), Hoechst-33342 (DNA), or Mitotracker Deep Red (mitochondria, MITO). A - frozen-thawed bull sperm. B - frozen-thawed rooster sperm. BF - bright field. Amnis ImageStream Imaging Flow Cytometer. Illustrative plots are shown.

limited capacity for biosynthesis and cell reparation. The differences in poultry sperm cells require specific protocols that provide the unique characteristics of sperm cells in this species (Çiftci and Aygün, 2018; Santiago-Moreno et al., 2016). Rooster spermatozoa are homogametic. The cells are approximately 0.5 μm at their widest point and approximately 70 μm in length (Chenoweth and Lorton, 2014). The filiform shape of the poultry sperm head is similar in diameter to the tail (Thurston and Hess, 1987). Therefore, sperm heads have relatively low cytoplasmic volume, and thus, a relatively low volume of cryoprotectants (**CPA**s) can be accumulated. There are technical limitations to sperm examination by computer-assisted sperm morphology analysis (**CASA**) systems, as these systems were designed to investigate the approximately spherically headed spermatozoa of mammalian species (Santiago-Moreno et al., 2016). For turkey and chicken spermatozoa, the neck region of the midpiece consists of a proximal and an elongated distal centriole. In contrast, guinea fowl spermatozoa contain only a single elongated centriole and associated pericentriolar projections (Thurston and Hess, 1987). Cross sections of the centrioles have the typical 'pinwheel' arrangement of nine triplet microtubules embedded in a cylindrical, dense wall (Thurston and Hess, 1987). The rooster spermatozoa midpiece contains approximately 30 mitochondria, with the mitochondria being integral in energy utilization for flagellar movement (Nguyen et al., 2014). Poultry sperm have a relatively long tail (between 90 and 100 μm; approximately 8 times longer than the sperm head) (Thurston and Hess, 1987) compared to bull spermatozoa (Bahr and Zeitler, 1964). For that reason, predisposes of poultry sperm seem to be more sensitive to freezing damage (Donoghue and Wishart, 2000).

Sperm cells mature in the testis and epididymis in most mammalian species. In roosters, as probably in other bird species, however, final maturation takes place in the vas deferens (the ducts that transport sperm from the epididymis to the ejaculatory organ) (Bernal et al. 2022).

It is known that the flagellar movement of sperm is driven by the sliding of microtubules with the motor protein dynein ATPase (Gibbons, 1988; Inaba, 2003). However, much is unresolved regarding the regulatory mechanism of movements in the axoneme. Reversible immobilization of chicken sperm motility (sperm suspended in a simple salt solution without Ca^{2+} were immotile at body temperature, but motility was instantly restored when the incubation temperature decreased below 30°C) has been described by Munro (1938) and Ashizawa and Nishiyama (1978). This reversible activation/inactivation of motility was also observed in drake sperm and partially in turkey sperm but not in quail sperm (Wishart and Wilson, 1999). In chicken sperm, motility inhibition at 40°C was quickly recovered when Ca^{2+} was included in the incubation mixture (Ashizawa et al., 1989). The depletion of Ca^{2+} from the medium by adding Ca^{2+} chelating reagents disturbed sperm motility even at 30°C. Therefore, it is considered that Ca^{2+} is essential for the maintenance of chicken sperm motility, as also reported in the sperm of many animals (Ashizawa et al., 1994b). Later, it was found that motility inactivation at body temperature was also cancelled by an increase in the intracellular pH (pH i) of the sperm (Ashizawa et al., 1994c). Sperm motility was activated in a 30°C temperature-controlled medium with various extracellular pH (pHe) values ranging from 7.3 to 10.1. In contrast, sperm exposed to 40°C medium, regardless of a pHe value lower than 8.1, were in a quiescent state (Ashizawa et al., 1994c). The pHi at 40°C was approximately 0.3 units lower than the pHi at 30°C. An acidic pHi at 40°C possibly plays a role in motility immobilization at body

temperature. The pH within the reproductive tract of the producing hen is generally higher than pH 8 (Fiser and Macpherson, 1974). Alternatively, the alkalization of sperm pHi is an important tool for sperm motility activation in chickens. Sperm acquire the potential for motility as they pass through the excurrent ducts (Ashizawa and Sano, 1990; Clulow, 1982; Howarth, 1983; Munro, 1938). Unfortunately, the question of how the composition of excurrent duct fluid affects sperm motility is not yet understood. In this regard, Ca^{2+} and HCO^{3-} have been identified as motility agonists for in vitro assays (Ashizawa and Sano, 1990; Ashizawa and Wishart, 1987).

Sperm drastically change their flagellar movement in response to the surrounding physical and chemical environment. Testicular sperm are immotile; however, they gain the competence to initiate motility during passage through the male reproductive tract. During the process of ejaculation, the sperm is activated and promptly initiates motility. Unlike mammals, ejaculated sperm in birds are stored in specialized tubular invaginations referred to as sperm storage tubules (SSTs), located between the vagina and uterus, before fertilization. The resident sperm in the SSTs are in a quiescent state and are then reactivated after release from the SSTs. It is thought that avian sperm can undergo a motility change from a quiescent to an active state twice (Matsuzaki and Sasanami, 2022).

Sperm storage within the female reproductive tract is important for maintaining fertility. After copulation, either via natural mating or artificial insemination, in female poultry species, that is, hens, turkeys, and birds, sperm are transported through the cloaca to the junction of the uterus and the vagina (uterovaginal junction [UVJ]) of the oviduct (Bakst, 1998) and in the infundibulum, although the primary storage site for sperm is the SSTs in the UVJ (Brillard, 1993). In the UVJ, sperm enter the tubular invagination sites of the surface epithelium of the mucosa, collectively called sperm storage tubules (**SST**s), where they are stored for longer periods, depending on the species, and retain their fertilizing capacity (Bréque et al., 2003; Holt and Fazeli, 2016). Uterus-vaginal protein extracts induce spermatic decapacitation in vitro (Camarillo et al., 2019). In general, the sperm storage tubules of hens are full for 24 to 48 h after insemination (Brillard, 1992; Bakst et al., 1994).

However, the molecular mechanism underlying sperm motility regulation is poorly understood (Matsuzaki and Sasanami, 2022).

In mammals, the acrosome reaction is required for sperm fertilization capacity, but thus far, there is no evidence that this process is necessary in birds (Howarth, 1970; Partyka and Niżański, 2022). Avian fertilization systems are quite different from those of mammalian species because of unique systems, such as polyspermic fertilization (Matsuzaki and Sasanami, 2022).

Another major difference between bovine and poultry reproductive biology that affects semen cryopreservation is the semen volume obtained per collection. The average ejaculate volume of a bull ranges from 5 to 8 mL, whereas semen volumes collected from poultry range from 0.1 to 1.5 mL. The rooster produces, on average, 0.6 mL of ejaculate (Taye and Esatu, 2022). Different roosters of the same species often produce different volumes of semen at different times (Getachew, 2016). The average volume ejaculated using the abdominal massage technique is approximately 0.25 mL (Gordon, 2005). The recorded semen volume was found to range from 0.37 to 0.73 mL (Peters et al., 2008). The average sperm concentration of poultry semen is much higher ($6-10\times10^9$ sperm/mL) than that of bull semen ($1-2\times10^9$ sperm/mL), and poultry sperm function is negatively impacted by excessive dilution, known as the dilution effect (Sexton and Fewlass, 1978; Sexton, 1981; Parker and McDaniel, 2006).

Sperm fertilizing ability is, however, drastically reduced at a dilution rate as high as 1:10 or 1:20 (Wilcox, 1958). It is possible that extensive dilution may destabilize the sperm membrane, which is detrimental to sperm motility and viability (Maxwell and Johnson, 1999). Furthermore, routinely collecting semen from donors (2−3 times a week) increases semen quality (Riaz et al., 2004).

In contrast to bulls, the pH of cockerel semen is higher, ranging from 7.2 to 7.6 (Getachew, 2016).

The biochemical characteristics and physiological roles of the various seminal plasma components in birds (carbohydrates, lipids, amino acids, hormones, and proteins) are poorly understood. Seminal plasma contains proteins expected to have an action on most cellular functions. A significant part of these proteins are common with the sperm cells, showing important exchanges between the sperm and their biological fluid. They include different metabolic functions, immunity, oxidoreduction actors and regulation, proteolysis, apoptosis, ion homeostasis, antimicrobial defences and epigenetic actors such as histones (Labas et al., 2014 ; Li et al., 2020). The variable amounts of many proteins are related to the different fertility capacities of poultry sperm. The role of seminal plasma in semen conservation (chilling and freezing) remains largely a matter of speculation, as both inhibitory and stimulating effects have been found (Santiago-Moreno and Blesbois, 2020).

The outermost membrane of the cell that surrounds the spermatozoon is called the spermatozoal plasma membrane (Chenoweth and Lorton, 2014). This membrane consists of a lipid bilayer made up of polar phospholipids, cholesterol, and proteins, a phospholipid-H_2O interface on the exterior of the cell, and the glycocalyx, the outermost region of the plasma membrane (Hammerstedt and Graham, 1992). Rooster sperm plasma membranes consist of many different phospholipid species (Bongalhardo et al., 2002), which are distinct from their mammalian counterparts. These membranes have a lower protein-to-phospholipid ratio of 0.46 (w/w) than bulls (0.80) and therefore are more resistant to cold shock when reduced to temperatures of approximately 5°C than mammalian spermatozoa (Wales and White, 1959; Pickett and Komarek, 1967;

Parks and Lynch, 1992). However, the rooster spermatozoal plasma membrane, while stable, is inelastic (Robertson, 1983), and when the sperm are exposed to hypotonic conditions, the membranes can easily rupture. This supports the belief that there is not enough research on the precise composition of sperm.

The resistance of sperm cells to cryopreservation has been verified by the membrane phospholipid content and fluidity (Holt, 2000). The fluidity of poultry sperm is closely related to the cholesterol/phospholipid ratio of the membrane (Holt, 2000) and is an indication of sperm freezability (Blesbois et al., 2005). It has been hypothesized that a high cholesterol content of the sperm plasma membrane is associated with a low freezing tolerance in mammals (Müller et al., 2008), while in poultry, the lower freezing tolerance of sperm cells is associated with lower concentrations of cholesterol and a lower fluidity of the plasma membrane (Blesbois et al., 2005). Membrane fluidity is dictated partially by the cholesterol: phospholipid ratio of the plasma membrane. Spermatozoon membranes with a high cholesterol:phospholipid ratio (rabbit 0.88; human 0.99) have more rigid membranes at room temperature. As these cells are cooled during cryopreservation, the high cholesterol content makes the membranes more fluid, and they incur less membrane damage than membranes from species that have low cholesterol:phospholipid ratios (rooster: 0.30; bull: 0.45) (Darin-Bennett and White, 1977; Parks and Lynch, 1992).

The plasma membranes of rooster sperm also contain a high level of unsaturated fatty acids in their phospholipid composition (Parks and Lynch, 1992). Poultry sperm cell membranes contain higher quantities of n-6 polyunsaturated fatty acids, including arachidonic (20:4n-6) and docosatetraenoic (22:4n-6) acids, than the membrane of mammalian sperm cells (Amini et al., 2015a). This makes poultry sperm cells extremely vulnerable to lipid peroxidation (**LPO**) and reactive oxygen species (**ROS**) produced by the cellular components of semen during freezing (Nguyen et al., 2014). Experimental studies have shown that LPO causes irreversible damage to DNA and decreases sperm motility and fertility in the freezing/thawing process (Morris et al., 2011; Amini et al., 2015a; Amini et al., 2015b). The amount of maintenance of the lowest possible percentage of respiratory oxidative stress in sperm: unlike oocytes, sperm are characterized by permanent movement and high-level metabolic activity (high respiratory rate) during a very short time (Clarke et al., 1984). Seminal plasma from numerous animal species, including poultry, normally contains antioxidant properties (Bréque et al., 2003; Carolina et al., 2006; Li et al., 2010; Partyka et al., 2012; Khan et al., 2012). However, antioxidant activity is dramatically decreased by cryopreservation, resulting in higher levels of LPO (Partyka et al., 2012).

Phospholipids, specifically their fatty acids and the cholesterol concentration of a membrane, affect the fluidity and osmotic tolerance of the membrane (Parks and Lynch, 1992). Rooster sperm have a lower limit of osmotic tolerance of 17 mOsm, although its upper osmotic limit has not been characterized (Watson et al., 1992). This critical osmolality of 17 mOsm for rooster sperm is defined as the osmolality at which 50% of the cells have swollen to the point of rupture of the plasma membrane. The critical osmolality of sperm differs between species, and the ability of sperm to survive these osmotic stresses and volume changes affect their ability to survive the freezing process (Hammerstedt et al., 1990). In theory, the rooster spermatozoon's cylindrical-tapered shape should accommodate larger amounts of cell volume change to a sphere shape without rupturing (Watson et al., 1992). In contrast, it has been observed that a rooster plasma membrane swells over both the head and tail (Bakst, 1980), and when one part of the membrane does not accommodate larger amounts of cell volume, this leads to the plasma membrane bursting sooner than theorized (Watson et al., 1992). Since the upper limit of osmotic tolerance for rooster sperm is unknown, both theories could prove true until this limit is uncovered.

Morris et al. (2011) found that a combination of intracellular protein content and osmotic shrinkage causes intracellular URF of spermatozoa during cooling, leading to osmotic shock when sperm are thawed and subsequent membrane damage. The sperm membrane is the first barrier for the sperm against the outside environment, and when this membrane is altered negatively, this can lead to an alteration of the sperm's intracellular environment and cell dysfunction. However, when the sperm plasma membrane is altered positively by increasing plasma membrane fluidity at lower temperatures, this in turn increases cell cryosurvival (Partyka et al., 2016). Therefore, focusing research on plasma membrane manipulation and alternative cryoprotectants is the key to improving rooster spermatozoa cryosurvival.

Semen Contamination of Bacteria

In poultry, pathogenic bacteria have been found to be associated with sperm, but their impact on sperm quality is not yet understood (Haines et al., 2013). Vizzier-Thaxton et al. (2006) demonstrated that *Campylobacter* could attach rooster sperm, which potentially results in decreased sperm quality. Bacteria can reduce broiler sperm motility upon exposure (Haines et al., 2013).

The bacteria possess a relatively high tolerance to the freezing procedure: the microbial contamination of cryopreserved semen stored for 6−35 years in liquid nitrogen was reported (Bielanski et al., 2003). For that reason, semen diluents containing compounds antibiotics with bactericidal or bacteriostatic properties should be used. On the other hand, the use of antibiotics is a highly controversial issue today due to the environment and antimicrobial resistance (Savvulidi et al., 2018). In addition, some antibiotics may be toxic to spermatozoa, as Morrel and Wallgren (2014) reviewed. Therefore, there is an urgent need to find alternatives to conventional antibiotics for use in semen extenders. Therefore, new approaches for the elimination of bacterial

contamination in semen were developed. Ozone treatment of semen (Gradil et al., 1995), using diode laser and light emitting diode on the bacterial contamination in semen (Hussein et al., 2008), or iron oxide (Fe_3O_4) nanoparticles have also been found to have antimicrobial activity against some bacteria (Guzman et al., 2012; Tsakmakidis et al., 2020; Tsakmakidis et al., 2021). Additionally, the use of antimicrobial peptides (AMPs) has become one of the most promising alternatives to the use of antibiotics in semen extender formulations to overcome the increasing bacterial resistance to antibiotics (Bussalleu et al., 2017).

Freezing Process

In the past 10 years, many studies have been performed to improve methods for bird sperm reproductive potential conservation after freeze–thaw cycles (Morris et al., 2011; Amini et al., 2015a; Partyka et al., 2016). During the poultry semen cryopreservation process (including the cooling−freezing and thawing procedures), osmotic and thermic shocks may cause damage to cell structure and metabolism (Long, 2006). Epigenetic modifications, which include DNA methylation and histone acetylation in rooster sperm, were also found to be significantly reduced after cryopreservation (Salehi et al. 2020). Therefore, works aimed at the improvement of the composition of diluents for cryopreservation, the selection of cryoprotectant and freezing methods (in straws or pellets), or the freezing protocols (low/fast) are still of research interest (Abouelezz et al., 2015; Nugrahini et al., 2019; Gatti et al., 2021; Tang et al., 2021). Different freezing and thawing procedures have been developed to prevent damage or destruction of cells during cryopreservation. These procedures are summarized in Table 1, including the results of viability and other functional parameters of sperm after thawing.

The first studies of preserving fowl semen used a slow cooling rate (Lake and Stewart, 1978; Sexton, 1981), for example, 1 to 10°C/min to −35°C. This method of freezing permits the flow of intracellular water from the cells to the outside the cells, thereby promoting extracellular crystallization and avoiding lethal intracellular ice formation. In slow freezing, low concentrations of CPA are used, the cells dehydrate during freezing, the temperature decreases slowly, and the formation of ice crystals is controlled. Currently, fast cooling rates are preferable (Váradi et al., 2013; Long et al., 2014), for example, 20 to 100°C/min. Optimal rapid freezing avoids excessive dehydration and shrinkage of cells. One of the possible methods is ultrarapid freezing (**URF**). High concentrations of CPA are used in URF, the cells are dehydrated before freezing, and the rate of temperature decrease is more than 1,000°C/min. During URF, the solution solidifies at a low temperature without crystals. However, high concentrations of CPAs could be toxic to cells (Dinnyes et al., 2007).

An interesting solution to sperm freezing seems to be gradual cooling by exposing the semen sample to liquid nitrogen vapor (Purdy et al., 2009; Rakha et al., 2017; Wu et al., 2020). Freezing semen packaged in straws over nitrogen vapor is a simple, quick, and inexpensive method that is widely used for cryopreservation of mammalian semen, even in commercial AI (artificial insemination) centers. This method has the great advantage of allowing the adoption of temperature gradients suitable for freezing semen in liquid nitrogen without the need for expensive dedicated equipment. In fact, the distance between the straw and the liquid nitrogen bath indirectly determines the thermal gradient during the freezing phase when the change from the liquid to the solid-state occurs. More rapid freezing rates are required to improve the survival of rooster sperm after cryopreservation, and a range of distances from 1 to 5 cm in nitrogen vapor above the surface of the liquid nitrogen is recommended for optimal sperm viability (Madeddu et al., 2016). Wu et al. (2020) as an optimal recommend exposing straws located as high as 6 cm above the liquid nitrogen surface. The methods used to control the freezing rate mainly include one-step freezing (Iaffaldano et al., 2016; Abouelezz et al., 2017; Fattah et al., 2017; Rakha et al., 2017) and 2-step freezing, that is, freezing slowly from 5°C to −35°C and then rapidly from −35°C to −150°C (Abouelezz et al., 2015; Blanch et al., 2014; Yang et al., 2016). A programmable freezing machine was also used (Santiago-Moreno et al., 2011; Blanco et al., 2012); however, it was not applied in practice, as this equipment is not always available (particularly in field conditions). Additionally, the temperature and method of thawing rooster sperm is one of the key points. During osmotic damage, the effect depends on the temperature. The formation of ice crystals is avoided during rapid warming, and extracellular thawing causes a sudden reduction in osmotic pressure, whereby the cryoprotectant is washed out before any toxic damage occurs (Wolfe and Bryant, 2001). It is difficult to compare the results of individual studies, as various papers have reported that the success of sperm cryopreservation procedures depends on the interaction among the type of cryoprotectant, semen freezing, thawing conditions, and packaging system used, each affecting sperm structure and function (Long et al., 2014; Iaffaldano et al., 2016). For example, the results of Miranda et al. (2018) indicate that the combination of EG (8%) and thawing at 5° C is the best option for rooster sperm. Compared to that, the authors Murugesan and Mahapatra (2020) , based on their research, recommend using cryoprotectant EG (8%) as well, however, with 37°C thawing temperature 37°C for 30 s. Mosca et al. (2020) investigated the effect of the CPA N-methylacetamide (**MA**) and of different thawing temperatures. The treatment that provided the best cryoprotective action and decreased the cellular cryodamage was the concomitant use of 6% MA and thawing at 5°C for 100 s. In contrast, Miranda et al. (2018) reported no differences comparing the effect of two thawing temperatures (5 vs. 37°C) on sperm motility of chicken semen cryopreserved with MA. Although very few studies have compared thawing temperatures in chicken semen, existing results indicate

Table 1.

Author	1	2	3	4 [%]	5	6	7	8 [%]	9 [%]	10 [%]	11 [%]	12 [%]	13 [%]
Abouelezz et al. (2017)	Lake and Ravie	S	Gly	8		−5 to −100°C at 10°C/min, −196°C	3min/5°C		57.8	31.3			
	Lake and Ravie	P	DMA	5		−196°C	60°C		57	46.4			
Abouelezz et al. (2015)	Lake and Ravie	S	DMA	6		5 to −35°C at 7°C/min; −35°C to −140°C at 60°C/min	30sec/37°C	21.1	21.3	10.8			
	Lake and Ravie	P	DMA	6		−196°C	60°C	18	19.8	12.8			
	Lake and Ravie	S	Gly	11		5 to −35°C at 7°C/min; −35°C to −140°C at 60°C/min	3min/5°C	47.6	43.4	2.1			
	Lake and Ravie	P	Gly	11		−196°C	60°C	18.6	24	4.2			
	Lake and Ravie	S	Gly	8		5 to −35°C at 7°C/min; −35°C to −140°C at 60°C/min	3min/5°C	37	39	28.8			
	Lake and Ravie	P	DMA	3		−196°C	60°C	23.6	24.7	25			
Appiah et al. (2020)	modified	P	Gly	13.5	Quercetin	−196°C	6sec/37°C						
Appiah et al. (2019)	modified	P	Gly	13.5	Melatonin	−196°C	30sec/37°C						
Behnamifar et al. (2021)	Lake	S	DMA	4		5 to −35°C at 7°C/min; −35°C to −140°C at 60°C/min	3min30sec/5°C	15.3	14.08				
	Lake	S	Gly	8		5 to −35°C at 7°C/min; −35°C to −140°C at 60°C/min	3min30sec/5°C	40.22	52.17				
	Lake + BSA	S	Gly	8		5 to −35°C at 7°C/min; −35°C to −140°C at 60°C/min	3min30sec/5°C	35.4	25.06				
Bernal et al. (2020)	Lake and Ravie + Val	S	Gly	8		5 to −35°C at 7°C/min; −35°C to −140°C at 60°C/min	3 min/5°C			32.6			
Blank et al. (2020)	Lake + FBS	S	DMF	6		5°C for 15 min, −35°C for 12 min, −135°C for 5 min, −196°C	5 min/5°C		29.62				
Ehling et al. (2012)	HS1	S	DMF + NMA 1:2	6,5/6,5		−3°C/min to −35°C, −50°C/min to −130° C at 60°C/min	4°C		44.3	81.1			
	HS1	S	DMF + NMA 1:1	6,5/6,5		−3°C/min to −35°C, −50°C/min to −130° C at 60°C/min	4°C		47.7	77.3			
	HS1	S	MA	6.5		−3°C/min to −35°C, −50°C/min to −130° C at 60°C/min	4°C		32.7	39.8			
Fattah et al. (2017)	BPSE	S	Gly	3		4 cm above the liquid N_2 for 7 min	30 sec/37°C		52.7				
	BPSE	S	Gly	3	L carnitin	4 cm above the liquid N_2 for 7 min	30 sec/37°C		69.1				
Feyzi et al. (2018)	Lake	S	Gly	5		5 cm above the liquid N_2 for 12 min	30 sec/37°C		61.2	48			
	Lake + NO	S	Gly	5		5 cm above the liquid N_2 for 12 min	30 sec/37°C		76.4	59			
Gatti et al. (2021)	Lake	S	DMA	3		3 cm above the liquid N_2 for 7 min	20sec/37°C		37.2		58.4	70.3	
	Lake	S	DMA	6		3 cm above the liquid N_2 for 7 min	20sec/37°C		42		53.7	74.6	
	Lake	S	DMA	9		3 cm above the liquid N_2 for 7 min	20sec/37°C		34.8		55.1	74.4	
	Lake	S	DMA	12		3 cm above the liquid N_2 for 7 min	20sec/37°C		32.4		50.7	65.8	
Li et al. (2020)	modified	P	Gly	13.5	Caragenan, CLC	−196°C	37°C						
Long et al. (2010)	BPSE	S	Gly	11		−20°C for 30 min, −196°C	2-3min/3°C	49.6					
Lotfi et al. (2017)	BPSE	S	Gly	3	HA	4 cm above the liquid N_2 for 7 min		46.1	55.3	65.5			57.5
Madeddu et al. (2016)	Lake	S	DMA	6		1cm/3cm/5cm/7cm/10 cm above the liquid nitrogen for 10 min	30sec/38°C	46.1	31.1				
Masoudi et al. (2021)	Lake	S			Mito-tempo	5 cm above the liquid N_2 for 12 min	30sec/37°C	65.1	62.3	65.3			87.3
Masoudi et al. (2019)	Lake	S	Gly	3	Glu	4 cm above the liquid N_2 for 7 min	30sec/37°C	60.8	58.8	63.8		49.8	91.4
Masoudi et al. (2018)	Lake	S	Gly	3	Co-Q10	4 cm above the liquid N_2 for 7 min	30sec/37°C	67.1	56.9	62.7		51.1	87.1
Mehaisen et al. (2020)	EK	P	DMA	6		4°C for 15 min (1.13°C/min), −196°C	6sec/60°C	32.5	34.7				64.6

(*continued on next page*)

Table 1 (*Continued*)

Author	1	2	3	4 [%]	5	6	7	8 [%]	9 [%]	10 [%]	11 [%]	12 [%]	13 [%]
Mehdipour et al. (2021)	Lake+AFP3	S	Gly	3.8		4 cm above the liquid N_2 for 7 min	30sec/37°C			61.5			
Mehdipour et al. (2018)	BPSE	S	SBL	0.5		4 cm above the liquid N_2 for 7 min	30sec/37°C	43.04	41.79			41.39	
	BPSE	S	SBL	1		4 cm above the liquid N_2 for 7 min	30sec/37°C	73.56	68.74	54		61.65	
	BPSE	S	SBL	1.5		4 cm above the liquid N_2 for 7 min	30sec/37°C	55.7	50.61			46.85	
	BPSE	S	EY	10		4 cm above the liquid N_2 for 7 min	30sec/37°C	56.76	53.76			47.71	
	BPSE	S	EY	15		4 cm above the liquid N_2 for 7 min	30sec/37°C	64.96	59.5			55.49	
	BPSE	S	EY	20		4 cm above the liquid N_2 for 7 min	30sec/37°C	76.03	70.12	60		66.26	
Mehdipour et al. (2020)	Lake	S	Gly	2	P188	4 cm above the liquid N_2 for 7 min	30sec/37°C			66.7			
	Lake	S	Gly	8	P188	4 cm above the liquid N_2 for 7 min				59			
Mphaphathi et al. (2016)	Kobidil+	S	DMSO	8		5 to −20°C at −1°C/min; 5 cm above the liquid N_2 for 5 min, −196°C	5min/5°C		46				
	Kobidil+	S	EG	8		5 to −20°C at −1°C/min; 5 cm above the liquid N_2 for 5 min, −196°C	5min/5°C		45				
	Kobidil+	S	PND	8		5 to −20°C at −1°C/min; 5 cm above the liquid N_2 for 5 min, −196°C	5min/5°C		21.8				
Mosca et al. (2016)	Lake	S	DMA	6		3 cm above the liquid N_2 for 10 min	30sec/38°C	46.9	35.8				
	Lake+T 0,1M	S	DMA	6		3 cm above the liquid N_2 for 10 min	30sec/38°C	46.4	38.4				
	Lake+S 0,1M	S	DMA	6		3 cm above the liquid N_2 for 10 min	30sec/38°C	44.7	35.3				
	Lake +S/T (0,1M; 0,1M)	S	DMA	6		3 cm above the liquid N_2 for 10 min	30sec/38°C	43.4	35.9				
Mosca et al. (2020)	Lake+T 0,1M	S	NMA	6		3 cm above the liquid N_2 for 10 min	100sec/5°C	50.7	52.3				
	Lake+T 0,1M	S	NMA	9		3 cm above the liquid N_2 for 10 min	100sec/5°C	36.6	35.5				
	Lake+T 0,1M	S	NMA	6		3 cm above the liquid N_2 for 10 min	30sec/38°C	22.8	20				
	Lake+T 0,1M	S	NMA	9		3 cm above the liquid N_2 for 10 min	30sec/38°C	20.5	18.1				
Miranda et al. (2018)	Kobidil+	S	DMA	6		5 cm above the liquid N_2 for 15 min	30sec/37°C		32.3				
	Kobidil+	S	DMF	7.5		5 cm above the liquid N_2 for 15 min	30sec/37°C		28.6				
	Kobidil+	S	MA	9		5 cm above the liquid N_2 for 15 min	30sec/37°C		30.7				
	Kobidil+	S	EG	8		5 cm above the liquid N_2 for 15 min	30sec/37°C		27.9				
	Kobidil+	S	DMA	6		5 cm above the liquid N_2 for 15 min	2min/5°C		31.1				
	Kobidil+	S	DMF	7.5		5 cm above the liquid N_2 for 15 min	2min/5°C		39.8				
	Kobidil+	S	MA	9		5 cm above the liquid N_2 for 15 min	2min/5°C		31.3				
	Kobidil+	S	EG	8		5 cm above the liquid N_2 for 15 min	2min/5°C		46.6				
Nabi et al. (2016)	Nabi	S	Gly	3		4 cm above the liquid N_2 for 7 min	3min/4°C		65.4	73.1		53.5	
	BPSE	S	Gly	3		4 cm above the liquid N_2 for 7 min	3min/4°C		21.4			21	
Najafi et al. (2021)	Lake+SBL	S	DMSO	4	P188	4 cm above the liquid N_2 for 7 min	30sec/37°C			79.5			
Najafi et al. (2019)	BPSE	S	Gly	3.8	Resveratrol	4 cm above the liquid N_2 for 7 min	30sec/37°C	70.8	64.3	58.1		64.5	
	BPSE	S	Gly	3.8	Resveratrol+NLC	4 cm above the liquid N_2 for 7 min	30sec/37°C	75	73	67.2		67.3	
Najafi et al. (2018)	BPSE	S	Gly	8	Lycopene	4 cm above the liquid N_2 for 7 min	30sec/37°C	69.7	64.08	57.8		59.22	
	BPSE	S	Gly	8	Lycopene LnL	4 cm above the liquid N_2 for 7 min	30sec/37°C	72.91	68.07	62.2		64.66	
Partyka et al. (2017)	EK	P	DMA	6	L-carnitin 1mM	−8°C for 15 min, −196°C	6sec/60 °c	37.6	48.06				
	EK	P	DMA	6	L-carnitin 5mM	−8°C for 15 min, −196°C	6sec/60 °c	34.33	49.94				
	EK	P	DMA	6	Hypotaurin 1mM	−8°C for 15 min, −196°C	6sec/60 °c	36.44	52.18				
	EK	P	DMA	6	Hypotaurin 10mM	−8°C for 15 min, −196°C	6sec/60 °c	32.83	51.4				
	EK	P	DMA	6	Taurin 1mM	−8°C for 15 min, −196°C	6sec/60 °c	40.18	53.58				
	EK	P	DMA	6	Taurin 10mM	−8°C for 15 min, −196°C	6sec/60 °c	35.41	53.37				

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Table 1 (*Continued*)

Author	1	2	3	4 [%]	5	6	7	8 [%]	9 [%]	10 [%]	11 [%]	12 [%]	13 [%]
Rakha et al. (2018)	RFE	S	EY	10		37°C to 4°C (−0.275°C/min), 4°C to −80° C (−8.4°C), −196°C	30sec/37°C	61.2	75.9				75.6
	RFE	S	EY	15		37°C to 4°C (−0.275°C/min), 4°C to −80° C (−8.4°C), −196°C	30sec/37°C	74.1	75	55.9			75.9
	RFE	S	EY	20		37°C to 4°C (−0.275°C/min), 4°C to −80° C (−8.4°C), −196°C	30sec/37°C	67.1	63.5				70.4
	RFE	S	EY	25		37°C to 4°C (−0.275°C/min), 4°C to −80° C (−8.4°C), −196°C	30sec/37°C	74.8	65.1				62
	RFE	S	Gly	20		37°C to 4°C (−0.275°C/min), 4°C to −80° C (−8.4°C), −196°C	30sec/37°C	61.9	51.8	46.5			65.6
Rakha et al. (2018)	modifided	S	Gly	20		37°C to 4°C (−0.275°C/min), 4°C to −80° C (−8.4°C), −196°C	30sec/37°C			53.1			
	modifided	S	DMSO	4		37°C to 4°C (−0.275°C/min), 4°C to −80° C (−8.4°C), −196°C	30sec/37°C						
	modifided	S	DMSO	6		37°C to 4°C (−0.275°C/min), 4°C to −80° C (−8.4°C), −196°C	30sec/37°C						
	modifided	S	DMSO	8		37°C to 4°C (−0.275°C/min), 4°C to −80° C (−8.4°C), −196°C	30sec/37°C			73			
	modifided	S	DMSO	10		37°C to 4°C (−0.275°C/min), 4°C to −80° C (−8.4°C), −196°C	30sec/37°C						
Salehi et al. (2020)	Lake	S	Gly	3		4 cm above the liquid N_2 for 5 min	30sec/37°C	60	77.2	59.3		32.9	
	BPSE	S	Gly	3		4 cm above the liquid N_2 for 5 min	30sec/37°C	51	68.3	47.2		35.5	
Murugesan and Mahapatra (2020)	Sasaki	S	DMA	6		4.5 cm above the liquid N_2 for 30 min	100sec/5°C	34.93		0			4.57
	Sasaki	S	DMSO	2		4.5 cm above the liquid N_2 for 30 min	100sec/5°C	22.66		0			94.85
	Lake	S	DMA	6		4.5 cm above the liquid N_2 for 30 min	100sec/5°C	34.46		0			4.14
	Lake	S	DMSO	2		4.5 cm above the liquid N_2 for 30 min	100sec/5°C	21.23		0			91.61
	Sasaki	S	EG	8		4.5 cm above the liquid N_2 for 30 min	100sec/5°C	23.72		0			94
	Lake and Ravie	S	EG	8		4.5 cm above the liquid N_2 for 30 min	100sec/5°C	26.52		0			92.33
	RFE	S	EG	8		4.5 cm above the liquid N_2 for 30 min	100sec/5°C	22.72		0.83			92.83
	Sasaki	S	EG	8		4.5 cm above the liquid N_2 for 30 min	30sec/37°C	26		18.39			84.58
	Lake and Ravie	S	EG	8		4.5 cm above the liquid N_2 for 30 min	30sec/37°C	24.67		48.12			69.98
	RFE	S	EG	8		4.5 cm above the liquid N_2 for 30 min	30sec/37°C	20.33		38.29			62.73
	Sasaki	S	DMF	6		4.5 cm above the liquid N_2 for 30 min	100sec/5°C	26		24.76			55.61
	Lake and Ravie	S	DMF	6		4.5 cm above the liquid N_2 for 30 min	100sec/5°C	23.81		30.89			22.03
	BPSE	S	DMF	6		4.5 cm above the liquid N_2 for 30 min	100sec/5°C	23.53		19.32			12.8
Shanmugam and Mahapatra (2019)	Lake and Ravie	P	DMF	6		−196°C	45sec/56°C	28.31		1.19			
	Lake and Ravie	P	DMF	9		−196°C	45sec/56°C	24.02		1.38			
	TES/NaCl	P	DMF	6		−196°C	45sec/56°C	17.79		0			
	TES/NaCl	P	DMF	9		−196°C	45sec/56°C	19.51		0			
	Lake and Ravie	P	DMA	6		−196°C	60°C	40.23		2.75			
	Lake and Ravie	P	DMA	9		−196°C	60°C	39.62		9.22			
	Lake and Ravie+S +BSA	P	DMA	6		−196°C	60°C	39.62		9.58			
	Lake and Ravie+S +BSA	P	DMA	9		−196°C	60°C	41.81		7.6			
	modifide	P	DMA	6		−196°C	60°C	38.71		9.81			
	modifide	P	DMA	9		−196°C	60°C	36.64		3.1			

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Table 1 (*Continued*)

Author	1	2	3	4 [%]	5	6	7	8 [%]	9 [%]	10 [%]	11 [%]	12 [%]	13 [%]
	RFE	S	NMA	12		4.5 cm above the liquid N_2 for 30 min	100sec/5°C	24.43		0			
	RFE	S	DMA	9		4.5 cm above the liquid N_2 for 30 min	100sec/5°C	27.85		0			
	RFE	S	DMSO	4		4.5 cm above the liquid N_2 for 30 min	100sec/5°C	27.21		1.9			
Shahverdi et al. (2015)	BPSE	S	Gly	2		5 cm above the liquid N_2 for 12 min	30sec/37°C		22.7				
	BPSE	S	LDL	1		5 cm above the liquid N_2 for 12 min	30sec/37°C		24.1				
	BPSE	S	LDL	2		5 cm above the liquid N_2 for 12 min	30sec/37°C		32.2				
	BPSE	S	LDL	4		5 cm above the liquid N_2 for 12 min	30sec/37°C		43.1				
	BPSE	S	LDL	6		5 cm above the liquid N_2 for 12 min	30sec/37°C		32.1				
	BPSE	S	LDL	8		5 cm above the liquid N_2 for 12 min	30sec/37°C		19.8				
Stanishevskaya et al. (2021)	LCM	P	DMA	6		−196°C	60°C		48	79			
	LCM+4,8mM	P	DMA	6		−196°C	60°C		48	82			
	LCM+9,5mM	P	DMA	6		−196°C	60°C		50	86			
Thélie et al. (2019)	Lake	S	Gly	11		−7°C/min	3min/4°C			83.3			
	BHSV	S	EG	10		−1°C/min	3min/4°C			1.5			
	BHSV	S	DMF	6		−15°C/min	3min/4°C			64.8			
	FEB	S	DMA	6		−60°C/min	5sec/40°C			35.3			
Wu et al. (2020)	Lake	S	Gly	2		−5°C/min, −9°C/min, −13°C/min, −6.5° C/min	3min/5°C			34.8			
	Lake	S	Gly	4		−5°C/min, −9°C/min, −13°C/min, −6.5° C/min	3min/5°C			75.1			
	Lake	S	Gly	6		−5°C/min, −9°C/min, −13°C/min, −6.5° C/min	3min/5°C			77.63			
	Lake	S	Gly	8		−5°C/min, −9°C/min, −13°C/min, −6.5° C/min	3min/5°C			70.09			
	Lake	S	Gly	11		−5°C/min, −9°C/min, −13°C/min, −6.5° C/min	3min/5°C			57.43			

1) extender (BSA- bovine serum albumine; Val-valine; FBS-fetal bovine serum; NO-nitric oxide; BPSE-Beltsville poultry semen extender; RFE-red fowl extender; TES-N-Tris-[hydroxymethyl]-methyl-2-amino-ethansulfonic acid; LCM- Leningrad cryoprotective medium; BHSV-Blumberger Hahnen Spermna Verdünner); 2) type of packaging (P-pellets; S-straws); 3) cryoprotectant (Gly-glycerol; DMA-dimethylacetamide; DMF-dimethylformamide; MA-methylacetamide; SBL-soyabean lecithin; EY-egg yolk; DMSO-dimethylsulfoxide; EG-ethylenglycol; PND-propandiol; LDL-low-density lipoproteins); 4) concentration; 5) antioxidant (CLC-cyclodextrine; HA-hyaluronic acid; Glu-glutathion; Co-Q10-coenzyme Q10; P188-poloxamer 188; NLC-nanostructure lipid carriers); 6) freezing rate; 7) thawing temperature; 8) viability; 9) total motility; 10) fertility; 11)plasmtic membrane damage; 12) mitochondrial activity; 13) acrosomal integrity

that thawing temperature may be a critical stage in the cryopreservation protocol.

Cryoprotectants

The survival of gametes after cryopreservation depends on the type and concentration of cryoprotectant (CPA) substances or solutions inserted into gametes before freezing. These are low- and high-molecular-weight organic substances readily soluble in water and are not particularly toxic even at higher concentrations. A number of these cryoprotectants are known today and are divided into freely penetrating CPAs (**P-CPA**s) and nonpenetrating CPAs (**N-CPA**s).

Thus, the important step in successful poultry semen cryopreservation is the choice of CPAs and their use during the process. Commonly used cryoprotectants are glycerol (**GLY**) (Wu et al., 2020), dimethylacetamide (**DMA**) (Abouelezz et al., 2017), dimethyl sulfoxide (**DMSO**) (Zhandi et al., 2017), ethylene glycol (**EG**) (Murugesan and Mahapatra, 2020), and dimethylformamide (**DMF**) (Rakha et al., 2020). These penetrating CPAs enter the cells and avoid intracellular water crystal formation and prevent damage caused by the excessive intracellular concentration of solutes (Holt, 2000; Pereira and Marques, 2008) and have been used across various protocols and extenders. Molecularly, previous CPAs are characterized by high permeability inside the cell and low toxicity compared to other CPAs (Mohammad et al, 2021).

CPAs have a toxic effect, the severity of which varies from one type to another. This effect will lead to a decrease in the various vital and morphological characteristics of the sperm in addition to the fertility capacity. The chemical toxicity of CPAs is associated with two effects. The first is the chemical action on the cells before cryopreservation, and the second is the cause of osmotic changes in the freezing solution. Low concentrations of CPAs are usually used during slow freezing. However, the concentration of CPAs used in URF is high. Therefore, it is necessary to consider the cell permeability for each cryoprotectant to estimate CPAs toxicity. High permeability can cause osmotic stress in cells before freezing and thawing processes. The rate of penetration of CPAs is also strictly temperature dependent. Therefore, the main factors influencing the toxicity of cryoprotectants are concentration, exposure temperature, and freezing time (Benesova and Trefil, 2015). Tselutin et al. (1999) focused their work on in vitro comparison of the three CPAs glycerol, DMA, and DMSO and showed that DMSO is the most toxic CPA and glycerol the least deleterious; the highest fertility rates were obtained with DMA, but only when spermatozoa were frozen in pellets. However, for example, for gene banking, which requires high levels of safety and clear identification, glycerol and straws are more convenient. Glycerol-treated spermatozoa unfortunately have a contraceptive effect after intravaginal insemination in chickens. More than 2% glycerol in an insemination dose causes complete absence of fertility (Neville et al., 1971; Lake, 1986). Moreover, Shanmugam and Mahapatra (2021) reported that when the concentration of glycerol is above 1% during insemination, it results in contraception, and no fertile eggs are obtained. This concentration is insufficient for sperm protection during cryopreservation. The problem may be solved by dialysis or centrifugation of the insemination dose after thawing to remove glycerol (Benesova and Trefil, 2015).

In recent years, N-methylacetamide (MA) has also become popular for the cryopreservation of rooster semen. The MA compound apparently has less cytotoxicity in cryopreserved cells (Mosca et al., 2016). When Sasaki et al. (2010) used a concentration of 9% MA, the overall hatchability rate was 89.5% in Yakido chickens. NMA displayed a negative concentration-dependent effect on fertility (Kim et al., 2014). Consequently, the related fertilization rate remains highly variable: in chickens, fertility after artificial insemination with frozen/thawed semen cryopreserved with NMA ranged from 0 (Shanmugam et al., 2018) to 100% (Sasaki et al., 2010), as Mosca et al. (2020) reviewed. N-Methylacetamide (NMA; H3C-C(O)-N(H)-CH3) is obtained by replacing the sulfinyl group [-S(O)-]in DMSO with an amide group [-C(O)-N(H)-] (Osuga et al., 2018). Because 6C, 7N, and 8O in the amide group have no d-orbital effects (Kauzmann, 1957) and the amide group is naturally involved in bioprocesses (Edsall et Wyman, 1958; Branden et Tooze, 1999), the cytotoxicity of NMA is lower than that of DMSO in cryopreserved human cells (Osuga et al., 2017). NMA is used in the cryopreservation of bovine spermatozoa (Jeyengran et Graham, 1980) and fowl semen (Sasaki et al., 2010; Lee et al., 2012). Polymers may be added to minimize the toxic effect of CPAs. These are nonpenetrating, remain in the space around the cells and at the same time reduce other CPAs (Partyka and Niżański, 2022). In recent years, work has also appeared on antifreeze proteins (**AFP**s) in the cryopreservation of poultry sperm. Unlike many other solutions, these proteins kinetically lower the temperature at which ice crystals form in a way that avoids heat shock. These proteins modify or prevent the growth of ice crystals and could stop recrystallization. This protects the cell membranes from cold-induced damage (Robles et al., 2019). Mehdipour et al. (2021) applied Type III antifreeze protein (AFP3) to the cryopreservation of spermatozoa from broiler breeder roosters, aiming to enhance post-thaw quality and fertility. The results suggested that AFP III may benefit sperm cryopreservation, considering post-thaw sperm quality and fertility parameters. Specifically, 1 mg/ml AFP III was the most appropriate concentration, with an optimal concentration potentially between 0.1 and 1 mg/ml. The high cost of AFP production can be a limiting factor for the widespread use of AFP in the cryopreservation process. However, studies have shown that AFPs can either protect or harm cells, depending on the AFP type and concentration used, other cryoprotectants present, and freeze–thaw protocols used (Brockbank et al., 2011;

Bang et al., 2013). Vitrification, in which a solution is cooled below the glass transition, is a procedure commonly used in cryopreservation to avoid problems with ice. Nevertheless, harmful ice formation is common during rewarming. Significant protective effects of AFPs after vitrification were noted, for example, in oocytes (Jo et al., 2011), sperm (Qadeer et al., 2015; Zilli et al., 2014), and embryos (Nishijima et al., 2014). The negative effects of AFPs seem to be related to the spicular shape of ice induced by fish AFPs (Rubinsky et De Vries, 1989; Ishiguro et Rubinsky, 1998), which fail to block ice growth out of the basal plane. Thus, other IBPs that bind multiple ice planes, including the basal planes, may be more promising for cryopreservation (Halwani et al., 2014).

Instead of using AFP, synthetic ice blockers (as a cost-effective alternative) might be used for sperm cryopreservation (De Leon et al., 2012; Quan et al., 2013).

The use of poloxamer 188 (P188) supplements seems promising in the cryopreservation process of poultry semen. P188 is an embryo cryopreservation supplement effective in many species and for cell lines and plant cells. Mehdipour et al. (2020) tested the suitability of P188 in the cryopreservation of rooster sperm, considering post-thawing motility, abnormalities, membrane functionality (hypo-osmotic swelling test), mitochondrial activity, viability, apoptosis status, reactive oxygen species production, and adenosine triphosphate (**ATP**) content after thawing and fertility and hatchability after AI. The results of this work proved that P188 could improve rooster semen cryopreservation and allow for the reduction of glycerol in extenders, with a consequent impact on the poultry industry. The positive effect of the addition of P188 extender on increased post-thaw quality and fertility of rooster sperm also proved Najafi et al. (2021).

In recent years, researchers have also focused on supplementing cryopreservation mixtures with proteins, such as fetal bovine serum (**FBS**), bovine serum albumin (**BSA**), and egg yolk (Rakha et al., 2018; Blank et al., 2020; Behnamifar et al., 2021). The outcomes of these works indicated that these proteins minimize some harmful effects of cryo-preservation, providing an alternative for chicken semen extenders.

Nonpenetrating CPAs (e.g., polyvinyl-pyrrolidone, sucrose, trehalose, raffinose, glucose, or lactose) act outside the sperm cell in the extracellular space and protect cells by dehydrating the intracellular space and limiting osmotic swelling during thawing (Rakha et al., 2020). Nonpenetrating CPAs, also known as osmoprotectants, are low-molecular-weight, hydrophilic, nontoxic molecules (Cleland et al., 2004). The addition of N-CPAs to the freezing medium serves to offset the cryodamage caused by P-CPAs. At similar concentrations, these substances are less toxic than P-CPAs, and they have multiple protective roles, such as inhibiting ice crystal growth and helping sperm stabilize internal solute concentrations under osmotic stress (Iaffaldano et al., 2016; Blank et al., 2020).

Following previous experiments, it is recommended that media contain at least one penetrating CPA and one nonpenetrating CPA. This mixture should be dissolved in phosphate or, preferably, N-(2-hydroxyethyl) piperazine-N-(2-ethanesulfonic acid (= HEPES)), which serves as a protective medium (Pereira and Marques, 2008; Dávila et al., 2015). The use of N-CPAs that act mainly as osmoprotectants could reduce the number of P-CPAs needed in sperm cryopreservation (Neville et al., 1971). Among the disaccharides, sucrose and trehalose are N-CPAs widely studied in different mammalian species: bulls (Chen et al., 1993; Foote et al., 1993), goats (Aboagla and Terada, 2003), boars (Gutiérrez-Pérez et al., 2009), and rabbits (Dalimata and Graham, 1997). In contrast, the effect of trehalose and sucrose on the post-thaw quality of poultry sperm has been poorly studied, and few reports are available. Bird sperm show specific characteristics that need added adaptations for sperm cryopreservation. They contain the same intracellular organelles as all amniotes, including mammals. However, in contrast to most mammals, they are filiform cells with a very low cytoplasmic content and a long flagellum adapted to their long trip in the female tract before reaching the site of fertilization (Thananurak et al., 2019). They consequently contain a relatively low amount of intracytoplasmic water and very high proportions of cellular membranes that make them highly sensitive to membrane injuries (Blesbois, 2012). Avian sperm are especially intolerant to volume and osmotic changes compared to mammalian sperm. Many sugars may play a role in the protection of sperm during the freeze–thaw process (Thananurak et al., 2019). For example, in avian species, the disaccharide sucrose showed contrasting effects on the success of sperm cryopreservation (Neville et al., 1971; Blanco et al., 2011; Blanco et al., 2012; Thananurak et al., 2019). In contrast, the disaccharide trehalose appears to be a very suitable cryoprotectant for avian sperm (Neville et al., 1971; Ehling et al., 2012; Stanishevskaya et al., 2021). Nevertheless, due to differences among the conditions used, the results reported in the literature are very variable. The addition of nonpermeating substances such as the amino acid glycine is beneficial for the cryosurvival of sperm. In rooster sperm, the inclusion of glycine into the diluent may improve sperm integrity by direct interaction with plasma membrane phospholipids (Cerolini et al., 2007; Gliozzi et al., 2011). Interesting results were obtained during the use of Ficoll (polysaccharide polymer) as a nonpermeating cryoprotectant for avian species. In a study by Miranda et al. (2018), the addition of Ficoll resulted in significantly higher post-thaw motility compared to trehalose, sucrose, or glycine. The effectiveness of Ficoll was attributed mainly to its capability of affecting solution viscosity, ensuring a greater stability of the sperm membrane and reducing mechanical stress and ice crystal formation, therefore increasing the ability to survive cryopreservation (Makarevich et al., 2008; Lagares et al., 2009).

Antioxidants

During cryopreservation, some sperm cannot take up antifreeze due to their small volume, causing the sperm to rupture and release ROS (reactive oxidizing species). In addition, cryopreservation increases ROS production in the freezing media, and ROS can attack the bis-allylic methylene group of plasma membrane phospholipids, leading to lipid peroxidation (**LPO**) (Hammerstedt et al., 1990; Li et al., 2010; Shahverdi et al., 2015). Therefore, avian sperm cells must be supplemented with additional antioxidants in the media to overcome the effects of lipid oxidation during freeze−thaw processes. For this reason, numerous substances with antioxidant effects, such as melatonin (Appiah et al., 2020; Mehaisen et al., 2020), k-carrageenan (**k-CRG**), CLC (cholesterol-loaded cyclodextrins) (Li et al., 2020), L-carnitine, hypotaurine, taurine supplementation (Partyka et al., 2017), Mito-TEMPO (Masoudi et al., 2021), Vit C, and Vit E (Amini et al., 2015a, Leao et al., 2021), and many others, have been tested. Seminal plasma from numerous animal species, including poultry, normally contains antioxidant properties (Hammerstedt et al., 1990; Watson et al., 1992; Li et al., 2010; Khan et al., 2012; Partyka et al., 2012).

The addition of antioxidants to extenders is highly desirable for both cooled and frozen rooster semen because they can reduce oxidative stress and improve fertilizing capacity. An overview of perspective antioxidants in poultry semen extenders was reviewed by Leao et al. (2021). This paper highlights glutathione, CoQ10 and L-carnitine for cooled semen, whereas for frozen semen, resveratrol, lycopene, and quercetin are most frequently used.

CONCLUSION

Currently, it is very important to improve the cryopreservation of rooster sperm for the protection of gene resources due to the occurrence of avian influenza. In cryopreservation, there are significant differences between mammalian and avian sperm cell morphology (Çiftci and Aygün, 2018). During freeze−thaw processes, sperm cells are exposed to damage such as oxidative stress, intracellular ice crystal formation during freezing and ROS accumulation (Leao et al., 2021; Masoudi et al., 2021; Partyka and Niżański, 2021). Moreover, rooster semen freezability depends on many factors, such as differences among the breeds and lines (Tselutin et al., 1999; Long, 2006; Long et al., 2010). As reported in various papers, the success of the sperm cryopreservation procedure may depend on the interaction among the type of cryoprotectant, semen freezing, thawing conditions and packaging system used, each affecting sperm structure and function (Long et al., 2014; Iaffaldano et al., 2016; Madeddu et al., 2016).

In developing the cryopreservation of poultry sperm, the main task is to improve it and simplify individual methods, including the freezing process, for a minimum period to integrate it into standard breeding practice without major demands on time and personnel.

For the widespread of cryopreservation of sperm in the poultry industry on a worldwide scale, the other very important task is to introduce novel methods and/or improve the interpretation of the results of currently used in vitro analytical methods (sperm quality tests). To this end, two aspects are essential: the ability of semen quality tests to precisely elucidate the molecular phenotype related to sperm quality and the ability of tests to predict the success of semen cryopreservation. Despite quite evident progress in the field (Blesbois et al., 2008; Labas et al., 2014), there are still a number of ambiguities with the correlation of the outputs of analytical methods and the actual fertilizing ability of rooster sperm. As an example of the essential necessity for the improvement of the results of currently used assays, the CellRox-based assay should be noted. It is broadly accepted that the CellRox dye stains only the cells with oxidative damage. Canonically, such CellRox-tagged cells are usually interpreted as damaged cells that are not of good quality. However, it was demonstrated recently that in equine sperm, the CellRox dye stains qualitatively good cells with healthy active mitochondria (Plaza Davila et al., 2015). According to our unpublished results, for chicken sperm, the situation is similar: CellRox dye stains viable cells with active mitochondria and not cells with oxidative damage. Until the interpretation of the CellRox-based assay will not be improved, researchers should be very careful with the interpretation of CellRox-based assay results.

Regarding freezability prediction, it should be concluded that no commonly accepted test is currently available, and future research needs to fill this gap. Today, the verification of sperm quality tests by artificial inseminations is still the only tool available for researchers. Therefore, this tool must be a part of each original paper dealing with the in vitro analytical methods.

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DISCLOSURES

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SUPPLEMENTARY MATERIALS

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