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Chapter

Cryopreservation of Domestic and Wild Animal Spermatozoa: Update of Knowledge

Diego A. Galarza, Mauricio Duma, Jorge X. Samaniego, Manuel Soria and Silvana Méndez

Abstract

Current sperm cryopreservation protocols for domestic and wild mammals aim to minimize the cryogenic damage caused by cell dehydration, ice formation, and osmotic stress. The optimization of sperm cryopreservation include the use of different synthetic and nonsynthetic-based extenders supplemented with additives (e.g., egg yolk, coconut water, etc.) and antioxidants (e.g., melatonin, L-carnitine, caffeine, resveratrol, etc.) that protect the plasmalemma, acrosome, and mitochondria against the detrimental effects caused by the cryopreservation process. Furthermore, the use of penetrating (e.g., glycerol, ethylene glycol, dimethylformamide, etc.) and nonpenetrating (e.g., sucrose and trehalose) cryoprotectant agents (CPAs) or their combination should be investigated to protect sperm during the freezing process in slow and ultra-rapid freezing procedures. Finally, new cryopreservation protocols should focus on freezing curves and initial cooling rates that allow optimal dehydration during freezing and adequate hydration during thawing. The suitable interaction of all these factors will allow a sperm subpopulation to survive cryopreservation with integrity and fertilizing capacity, contributing to the improvement of the efficiency of genetic resource management and the development of germplasm banks that support the preservation of genetic diversity in domestic and wild animals.

Keywords: cryopreservation, cryoinjury, additives, cryoprotectant agents, spermatozoa

1. Introduction

Sperm cryopreservation is a valuable procedure to preserve male fertility and its subsequent application in different assisted reproduction techniques (ART) such as artificial insemination (AI), *in vitro* fertilization (IVF), or even *intracytoplasmic sperm injection* (ICSI). Semen cryopreservation also is used to facilitate long-term storage and transport. Another well-defined purpose that sperm cryopreservation has is the conservation programs of domestic and wild species. In the domestic species, the application of reproductive biotechnologies with cryopreserved gametes is aimed at genetic improvement and increased fertility. However, wild animals allow the genetic

material of these endangered species to be conserved indefinitely until its proper use. In this sense, implementing cryogenic banks that preserve sperm from wild animals should be a governmental strategy for the (*ex-situ*) conservation of these species.

Although the first cryopreservation studies were carried out using dry ice (at −79°C), the widespread use of liquid nitrogen (LN_2) since the 1950s has made it the substance of choice for cryopreservation of genetic material and its storage at −196°C. The discovery of the cryoprotective role of glycerol (GLY) by Polge et al. [1] has allowed for several advances in the cryobiology of different cells and tissues, especially sperm, oocytes, and embryos.

Other applications of semen freezing technology have been developed in various contexts, including (a) cryopreservation of genetic resources and biomedical research in fish; (b) storage of mouse, rat, and primate sperm for biomedical research purposes; (c) the establishment of semen banks for the conservation of regional and national breeds; and (d) the establishment of sperm banks to support conservation breeding programs [2–5].

Nowadays, sperm cryopreservation involves three well-established processes: cooling, freezing, and vitrification. The highest fertility rates have been achieved with fresh semen after AI. Cold-stored sperm produces better fertility results than frozen-thawed or vitrified-warmed semen. Spermatozoa stored in liquid at 5–15°C decrease the cell metabolic rate and ATP generation. Nevertheless, sperm quality and fertilizing ability decrease as storage time increase [6]. During the freezing process, however, sperm are susceptible to various stresses during 'supercooling' and 'freezing' events, which cause several ultrastructural, biochemical, and functional changes, resulting in cryodamage in some sperm subpopulations. Typically, no more than 50% of sperm survive cryopreservation, resulting in low fertility rates [7, 8]. Furthermore, the remaining surviving spermatozoa may have damaged organelles and membranes, reducing biological efficiency (e.g., sperm capacitation and acrosomal reaction) [9]. Cryoinjuries produced during the freezing-thawing process can be due to factors related to thermal shock (i.e., cold shock), ice formation, dehydration, increased solute concentration, and osmotic shock. Cold shock affects spermatozoa, and its negative effects are associated with irreversible changes in sperm capacitation, damage to the plasma membrane, induction of the caspase cascade involved in apoptosis, hypomethylation, and DNA fragmentation, as well as low fertilizing capacity. All of the aforementioned events are influenced by cooling rates during the cryopreservation process [10–12].

Cryoinjuries that occur during cryopreservation mainly include membrane rupture, denaturation and/or displacement of plasma membrane proteins, degeneration of the mitochondrial membrane, and acrosomal reaction. Likewise, the freezing-thawing process leads to a breakdown of the microtubules that make up the flagellum of the spermatozoon, resulting in reduced motility. Membrane and microtubule alterations are caused by osmotic damage that occurs during the freezing-thawing process [13] (**Figure 1**). Another important deleterious effect of cryopreservation is osmotic stress, which can also cause variations in the dimensions of the sperm head [14]. It has been suggested that over-condensation of sperm chromatin, damage to the plasma membrane and acrosome, as well as acrosome loss and damage to the cell cytoskeleton, could cause a reduction in the size of the head [15–17].

Furthermore, oxidative stress is associated with lipid peroxidation (LPO), which leads to a reduction in sperm quality. The high concentration of polyunsaturated fatty acids (PUFA) in the sperm membranes of mammals (e.g., ram, horse, and dog) makes them very vulnerable to oxidative damage [18, 19]. Mammalian spermatozoa

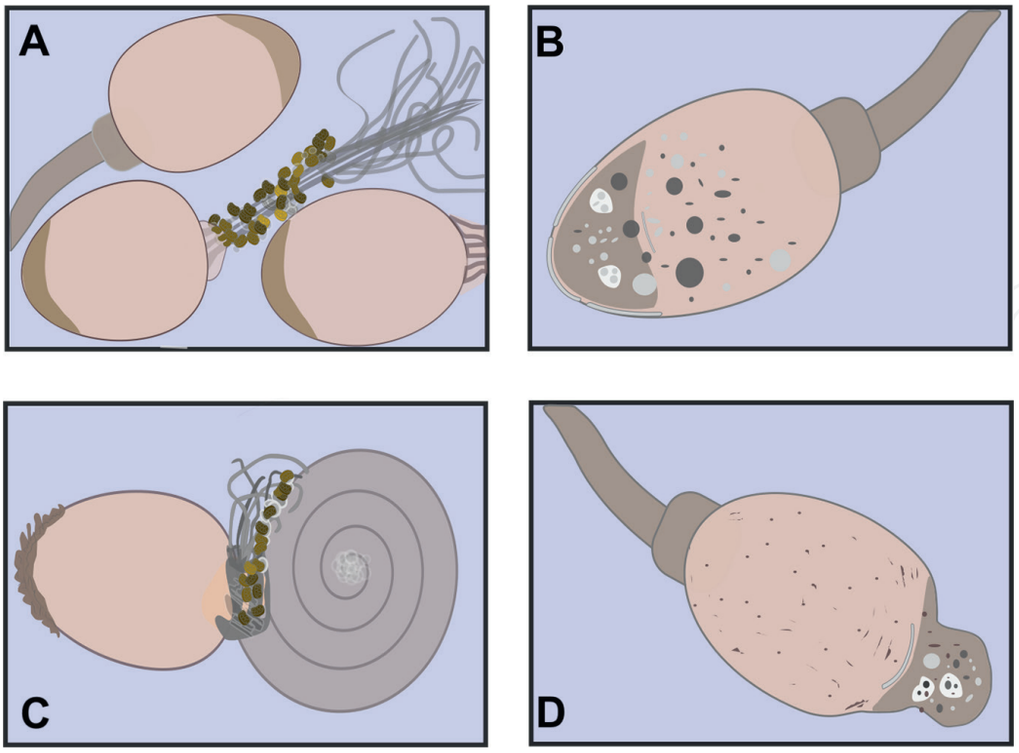

Figure 1.
Cryogenic damage is caused by the freezing and thawing process. Swollen midpiece damaging the mitochondria (A). Disruption of the perforated acrosomal membrane (B). The ridge of the acrosome membrane wrinkled, the tail rolled, and the midpiece broken (C). The apical ridge of the acrosome is somewhat swollen (D).

are also vulnerable to attack by free radicals (e.g., H_2O_2, O_2- and OH-) since they are rich in PUFAs. ROS readily combines with PUFAs, leading to excessive production of LPO. It is known that the increase in ROS during cold storage alters the mitochondrial and plasmatic membranes of sperm, thereby altering their motility and causing DNA fragmentation [20–22]. These cellular alterations decrease the viability, kinematic variables, and fertilizing capacity of cryopreserved spermatozoa.

In this sense, current cryopreservation protocols aim to minimize cryogenic damage to sperm and reduce alterations. Therefore, the following chapter review focuses on the optimization of cryopreservation through the analysis of freezing ramps (cooling rates), cryoprotectant agents, additives, and alternative cryopreservation procedures (e.g., ultra-rapid freezing or kinetic vitrification).

2. Basic principles of cryopreservation and cell cryoresistance

Sperm cryopreservation still constitutes a technological challenge due to the low cell survival rates obtained, and consequently, the low fertility rates achieved after AI [21]. Several advances in human, ram, bull, dog, and even rodent sperm cryopreservation have been reported [23–26]. However, in other species (both domestic and wild), progress has been limited. The general theory of cryobiology and the basic principles of understanding cell survival during freezing and thawing have been detailed. Cell cryosurvival is based on water transport through membranes and the interactions between solutes, cryoprotectant agents, temperature decrease, and the integrity and composition of sperm membranes. These events are determined by

the cooling rates to which the cells are subjected from the start of freezing until they reach the solidification stage (< −80°C).

During a temperature drop, the sample water remains in a liquid state, and the solution undergoes supercooling. The '*seeding*' process, equivalent to the first point of ice formation, occurs randomly at some point below the freezing point (between −8 and − 14°C [27, 28]). The randomness of this stage is dictated by the probability that groups of water molecules will organize themselves into groups and start a chain reaction. Since this is an exothermic reaction, enough heat is released to raise the temperature of the sample significantly. Depending on the samples being frozen (in straws of 0.25 or 0.5 ml) and the freezing protocol (one-, two-, or three-step cooling rates by acceleration or deceleration), the temperature may remain static for a short time before cooling resumes or may even result in a transient rise in temperature before cooling resumes. This event is known as the dissipation of the latent heat of fusion. These temperature variations always occur when plastic straws with sperm samples are freezing conventionally by exposing the straw horizontally to LN_2 vapors at different heights above the level of LN_2. Researchers have been divided as to whether this process is harmful or not.

When ice begins to form during the cooling process, dissolved salts, and other compounds are squeezed out of the ice and become concentrated in the ever-shrinking spaces between the developing ice crystals. Cells interact osmotically with these unfrozen pockets (*lakes*) of high solute concentration, and water tends to be drawn from the cells, resulting in cell shrinkage (decreased head dimension due to cell dehydration). This hypertonic content is called "*veins*" in some literature, as determined by Cryo-SEM (Scanning Electron Microscopy) [29]. This process results in two counteracting deleterious effects: 1) if the cooling rate is slow, cell membranes are exposed for longer periods of time to pockets of hypertonic solutions, with possible deleterious effects such as protein and lipid extraction and ROS generation; conversely, 2) if the cooling rate is high, cells are cooled rapidly, their cytoplasmic water

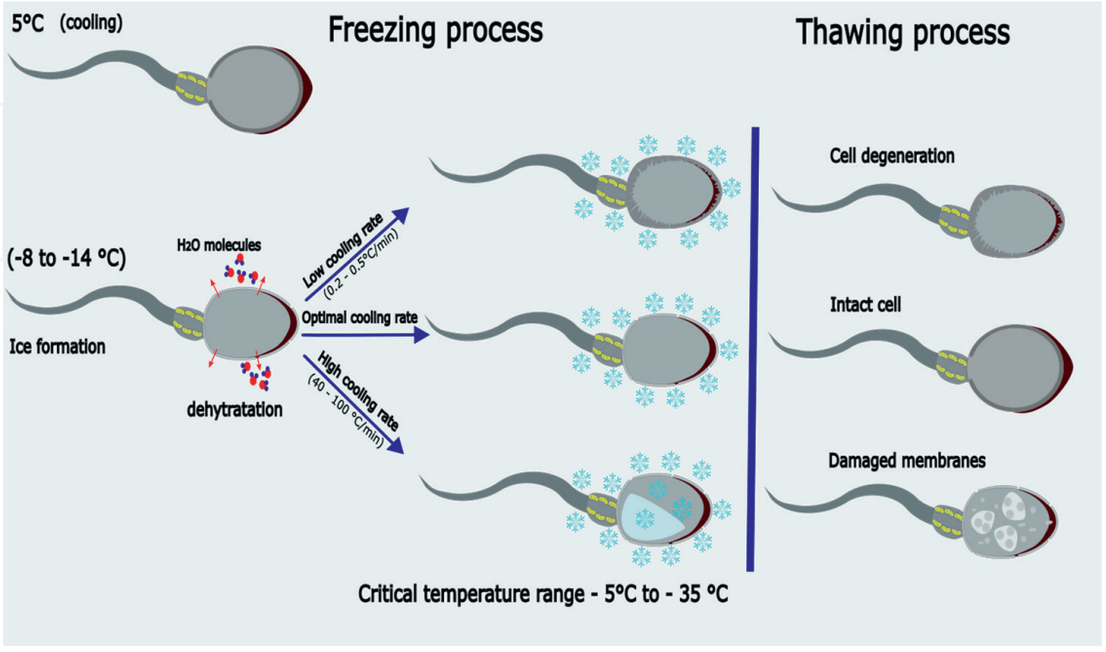

Figure 2.
Scheme of the physical events that occur in the sperm during the freezing process using either low, high, or optimal cooling rates. In addition, the state of integrity or cell damage caused to the sperm after thawing is shown.

content remains high, and they risk lethal intracellular ice formation. In practical terms, the optimal cooling rate should be considered as a compromise between these opposing effects (**Figure 2**).

Cooling rates determine cell survival, especially in the initial stage of freezing. During the critical temperature range (−5°C to −25°C), the cell suffers dehydration that can be severe or minimal if the cooling rates are very slow or high, respectively. Both low or high cooling rates cause cell degeneration and death. Cooling rates are important for the survival of sperm. As extracellular ice formation takes place, cells and dissolved salts are excluded from the ice and become concentrated in the unfrozen fraction that remains between the growing ice masses. Therefore, the osmotic force of the nonfrozen fraction increases, causing an efflux of water from the cells, resulting in cellular shrinkage [30]. These shrunken cells can survive thawing, but locomotion is undesirable due to flagellum contraction.

Another major factor is the exposure of spermatozoa to hypertonic solutions (with a high salt content) during the dehydration process before freezing. These observations indicate that spermatozoa interact with ice crystal regions and regions of high salt concentration and that this level of heterogeneous distribution is established around the time of freezing and maintained until the sample is thawed. As a result, most spermatozoa remain trapped in regions of high solute concentration (i.e., into the veins) separated by regions of pure ice (i.e., lakes) [31–33] (**Figure 3**).

In addition to the purely osmotic interactions associated with freezing and thawing processes, cell membrane lipids undergo partially irreversible phase transitions [34], resulting in the appearance of semi-crystalline lipid matrices as the temperature decreases during cooling and freezing. This event is believed to be responsible for the damaging effects of 'cold shock,' which occurs when spermatozoa are rapidly cooled in the absence of cryoprotective additives. Cold shock is not only recognizable due to the structural damage caused to plasma and acrosomal membranes, but also induces loss of cellular homeostasis through inadequate membrane permeabilization, excessive calcium uptake, and uncontrolled loss of potassium [35].

Currently, several factors are known to determine sperm cryoresistance during cryopreservation. The lipid composition of the membrane is a key factor that determines sperm cryoresistance. Species with high cholesterol/phospholipid ratios are the ones that best resist cooling processes (e.g., rabbit sperm) [36]. Similarly, the composition of seminal plasma directly influences the sperm's response to freezing processes. In this sense, proteins related to better freezability, and others that seem to predispose to greater damage, have been identified [37]. Long-term exposure to proteins originating in the bull's seminal vesicles, generically known as BSP (major proteins of bovine seminal plasma), predisposes to major cell damage. BSPs cause an efflux of cholesterol and phospholipids from the bull spermatozoon membrane, making it more sensitive to cryopreservation processes. The amino acid profile of the seminal plasma can also contribute to variations in cryoresistance, with variations in the profile possibly influenced by a genetic (breed) component.

The origin of spermatozoa also determines variations in resistance to cold shock. Ejaculated spermatozoa are more susceptible to cold shock than epididymal spermatozoa [14]. In this sense, it has been previously reported that sperm from the epididymis appears to have more cryoresistance than sperm from ejaculates, with differences in membrane composition influencing the equilibration time required during cryopreservation [38–40].

Circulating testosterone and prolactin levels, which are generally determined by the mating season in species with seasonal reproductive activity, can also confer

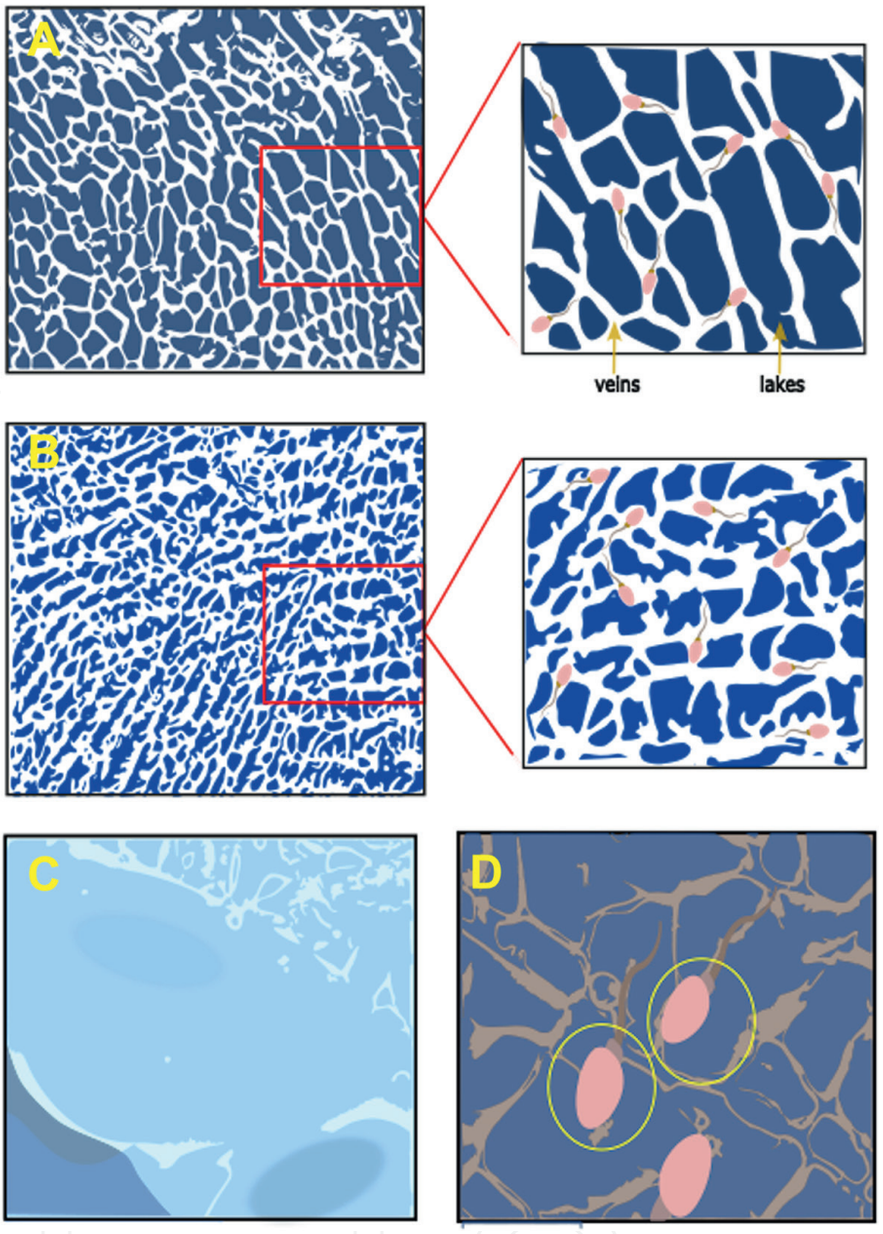

Figure 3.
Schematic representation of the distribution of ice crystals within a conventional slow-freezing matrix using 5% glycerol (A), and ultra-rapid freezing using 100 mM sucrose contrasted with a vitrification matrix (without ice crystals). The blue arrows show the veins (highly concentrated solution) and lakes (ice). Spermatozoa are displaced and trapped within the veins during freezing (D).

morpho-functional changes in the spermatozoa that make them more or less sensitive to cryopreservation [41]. Recently, it has been determined that the concentration of circulating testosterone in the Iberian ibex (*Capra pyrenaica*) appears to influence the cryoresistance of spermatozoa. This confirms that observed seasonal changes in sperm freezing occur in some wild ruminant species (e.g., mouflon and ibex), irrespective of fresh sperm quality [42].

Aquaporins (AQPs), which are proteins of the sperm membranes related to water diffusion, determine the cell cryoresistance of domestic and wild animals during freezing and thawing processes. It has recently been reported that transmembrane AQPs have potential use as cryotolerance markers in mammalian spermatozoa [43]. Thirteen AQPs have been identified in mammals and are divided into three families

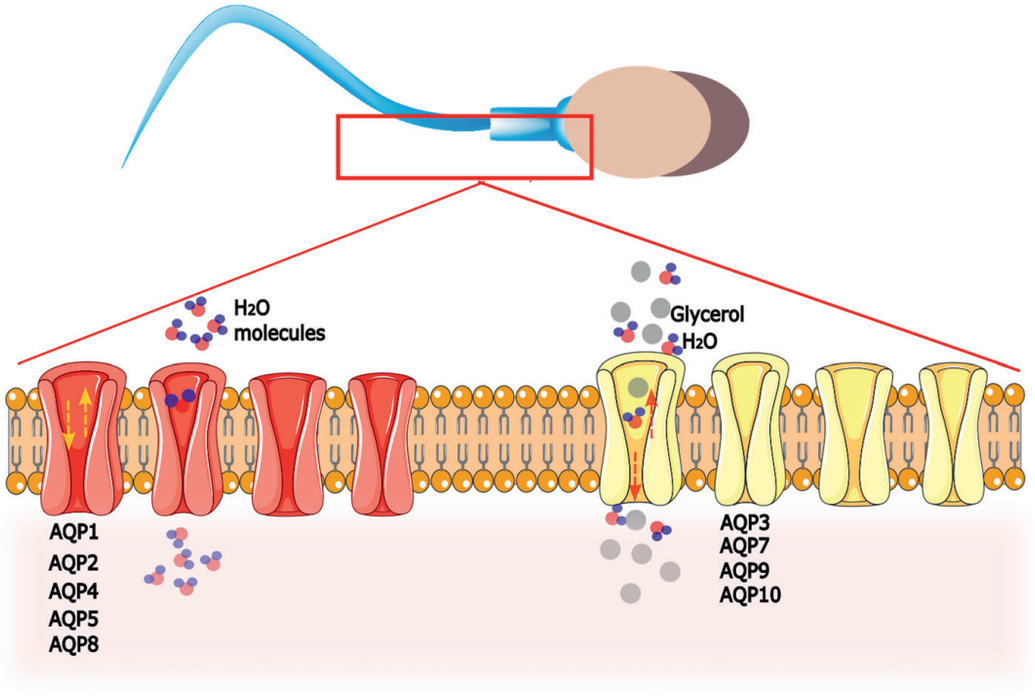

Figure 4.
Mechanism of flow and efflux of water-glycerol in the spermatozoa mediated by the transmembrane proteins aquaporins (AQPs).

based on their functionality: 1) the first family includes AQP1, AQP2, AQP4, AQP5, and AQP8, which selectively transport water; 2) the second family transports water and glycerol (aquaglyceroporin), and the latter acts as an energy substrate during sperm maturation and storage and includes AQP3, AQP7, AQP9, and AQP10 [44, 45]; finally, the third family is called the super aquaporins, which include AQP11 and AQP12 [46] (**Figure 4**). The presence and localization of AQPs in spermatozoa vary between species and cell regions. AQP3 has been identified in bull [47], boar [48], stallion [49], mouflon and ibex [50], dromedary camel [51], mouse, and human [52] sperm. In sperm cryopreservation, it has been suggested that the flow of water and cryoprotectants through the sperm membrane during the freezing and thawing process is regulated, to a large extent, by aquaglyceroporins. The role of AQPs in the regulation of mammalian sperm motility is not well known. However, it is reasonable to assume that they are involved in the activation of spermatozoa during hypotonic stress once they are released from the epididymis and come into contact with seminal plasma [36]. In porcine spermatozoa, AQP11 content appears to be correlated with motility and membrane integrity [47]. On the other hand, it has been hypothesized that differences in their expression in the sperm cell membrane, even at the individual level, could explain a better or worse response observed in specimens of animals known as good or poor freezers (e.g., horses).

3. Freezing ramps and cooling rates

Freezing ramps used in sperm cryopreservation, either through a cryogenic box (cryo-box) or through a programmable freezer (bio-freezers), are key factors for achieving successful cell survival. As mentioned earlier, both extremely high and/or

slow cooling ramps have detrimental effects on sperm cells [53]. The characteristics and shapes of ice crystals depend on the cooling rates, mainly extracellular ones, which are one of the principal causes of cell damage during the freezing process. The rate at which ice crystals form during sperm cryopreservation is highly dependent on the freezing protocol. Extracellular ice or changes in the composition of the external solution (diluent or freezing medium) caused by the conversion of water to ice are thought to be a cause of sperm damage during the cryopreservation process [10]. Furthermore, the size and shape of ice crystals and ice masses depend on the cooling rate. Therefore, the optimal cooling rate should be expected to be a compromise between these opposing effects mentioned above to cause minimal cryoinjury and consequently achieve desirable sperm viability after thawing. Higher cooling rates result in smaller crystals (61), and even ultra-rapid cooling rates result in extremely small crystals compared to ice crystals from conventional freezing [54] (see **Figure 3**). In work carried out by Bóveda et al. [29], it was determined by Cryo-SEM that the ice crystals were smaller as the cooling rates were higher; they even determined that ice crystals did not form in kinetic vitrification.

During the freezing process, the cooling rate of the sample inside the straw (non-frozen sample) is not constant due to the dissipation of the latent heat of fusion [27]. Therefore, the freezing rate inside the straw may be different than what is expected by the programmable biological freezer due to the heat generated by the ice formation. The cooling rate immediately after the dissipation of the latent heat of fusion can be higher than the programmed cooling rate [55]. Conventional slow freezing by static LN_2 vapors contained in Styrofoam cryo-boxes produces a high initial cooling rate in the freezing phase during and after ice nucleation. These conventional slow-freezing methods produce cooling rates by deceleration (initially at 40°C/min, followed by 17°C/min, and finally 3°C/min). The high initial cooling rates are achieved by exposing the straws horizontally in a rack freezing ramp (ramp) at a distance of 4 to 5 cm from the LN_2 surface [56, 57]. It has been identified that the initial cooling rate of conventional slow freezing is approximately 40°C/min, and at some point in that rapid temperature drop, ice nucleation occurs (less than −20°C) [27, 28]. These high cooling rates could cause cold shock damage and intracellular ice crystal formation, which can be lethal to cells. Previous studies suggest that the use of static LN_2 vapors is more harmful to freezing sperm (e.g., ram, stallion, bull, etc.) in terms of sperm integrity and functionality than programmable freezing methods with initially low cooling rates (e.g., 4 to 5°C/m) in the critical temperature range where ice nucleation occurs (−5°C to −35°C) [58, 59].

Mammalian sperm freezing protocols have been modified from their conventional processes using LN_2 vapors by improving their cooling rates, as determined by Woelders and Chaveiro (65). These authors developed a theoretical prediction of optimal freezing rates and determined that using slow initial cooling rates before and during ice nucleation, and then accelerating to high cooling rates (60–140°C/min) up to −80°C, would allow for better sperm cryosurvival. Based on this prediction, several investigations have been carried out using conventional freezing with cooling rates that include acceleration, starting with slow initial cooling rates followed by higher cooling rates.

Conventional slow freezing that uses accelerating cooling rates has been developed in several domestic and wild species. For example, promising survival results were obtained in bulls' sperm when a protocol was used that included an initial cooling rate of 10°C/min from +4°C to −10°C, followed by 40°C/min from −10°C to −150°C

[60]. In goat semen, a triphasic ramp yielded good results of motility and viability after thawing: from +4°C to −5°C at 4°C/min, then from −5°C to −110°C at 25°C/min, and finally from −110°C to −140°C at 35°C/min; once the temperature of −140°C has been reached, the straws are immersed in LN_2 (−196°C) [61]. In rams, the application of various freezing ramps and interaction with GLY concentration has given variable results [62, 63]. Recently, a biphasic ramp from +5 to −10°C at 5°C/min, and from −10 to −130°C at 60°C/min was used, which allowed good preservation of the kinetic variables, as well as the integrity of the plasmatic, mitochondrial, and acrosomal membranes, and less DNA fragmentation compared to the conventional freezing protocol [27, 28]. Likewise, Esteso et al. [57] demonstrated better cryosurvival results of spermatozoa from wild ruminants 'Iberian ibex' (*Capra pyrenaica*) when using a triphasic acceleration freezing ramp (4°C/min, −25°C/min, −35°C/min) compared to conventional freezing using a Styrofoam cryo-box that recorded cooling rates by deceleration: 40°C/min from +5°C to −35°C, followed by 17°C/min from −35°C to −65°C, and finally 3°C from −65°C to −85°C. In all cases, both in domestic and wild species, better results have been obtained through freezing protocols with cooling rates by acceleration (gradually increase) compared to those freezing protocols with high initial cooling rates followed by decelerating cooling rates (e.g., those obtained with the conventional method in LN_2 vapors). Cryopreservation of ferret (*Mustela putorius furo*) sperm by decelerating and accelerating freezing rate protocols showed that using an initial cooling rate of 5°C/min from 5°C to −10°C, and then accelerating to 60°C/min from −10°C to −130°C at 60°C/min yielded better post-thaw sperm viability and acrosome integrity than freezing rate by deceleration: 40°C/min from 5°C to −35°C, then decelerating to 17°C/min from −35°C to −65°C, and finally to 3°C/min from −65°C to −85°C [64].

Different rates of semen freezing have been studied in rams and other ruminant species, with variable results. Kumar et al. [65] recommended a controlled linear cooling rate of 30°C/min for ram semen during the ice nucleation and the critical temperature range (−5 to −25°C). Additionally, Fang et al. [66] and Vichas et al. [59] found relatively low optimal cooling rates (25 to 40°C/min) for ram sperm. However, there are studies that suggest that higher cooling rates are acceptable or better for ram [62] and bull [67] semen, contradicting the previous findings.

Recent studies on slow freezing of spermatozoa from guinea pigs, dogs, and horses have used a two-ramp freezing system. A Styrofoam cryo-box measuring 30 × 29 × 31 cm in length, width, and height, respectively, and including two freezing ramps at two heights above the LN_2 level has produced desirable results for motility and plasma membrane integrity. In fact, some of these have generated initially slow cooling rates and then higher rates (through acceleration) (**Figure 5**). A recent study using this Styrofoam cryo-box with 1.7 liters of LN_2 reported on the freezing of epididymal guinea pig spermatozoa with two freezing ramps at two heights of 24 and 10 cm above the LN_2 level [68]. Sperm samples inside 0.25 ml straws were exposed to LN_2 vapors for 3 and 2 minutes on each ramp at 24 and 10 cm, respectively. In this protocol, the internal temperature (T_{in}) of the straw was recorded using a digital thermometer with a type K thermocouple sensor (fine wire) that determined the ice nucleation occurring at −11.2°C, and the dissipation of the latent heat of fusion lasted for 27 seconds (**Figure 6A**). This freezing protocol caused decelerating cooling rates: an initial rate of 39°C/min before and during ice nucleation, which decreased to 15°C/min. Despite drastically decreasing the sperm quality variables, a cryosurvival of 11.2 ± 1.41% of total motility and 14.8 ± 0.70% of simultaneous integrity of

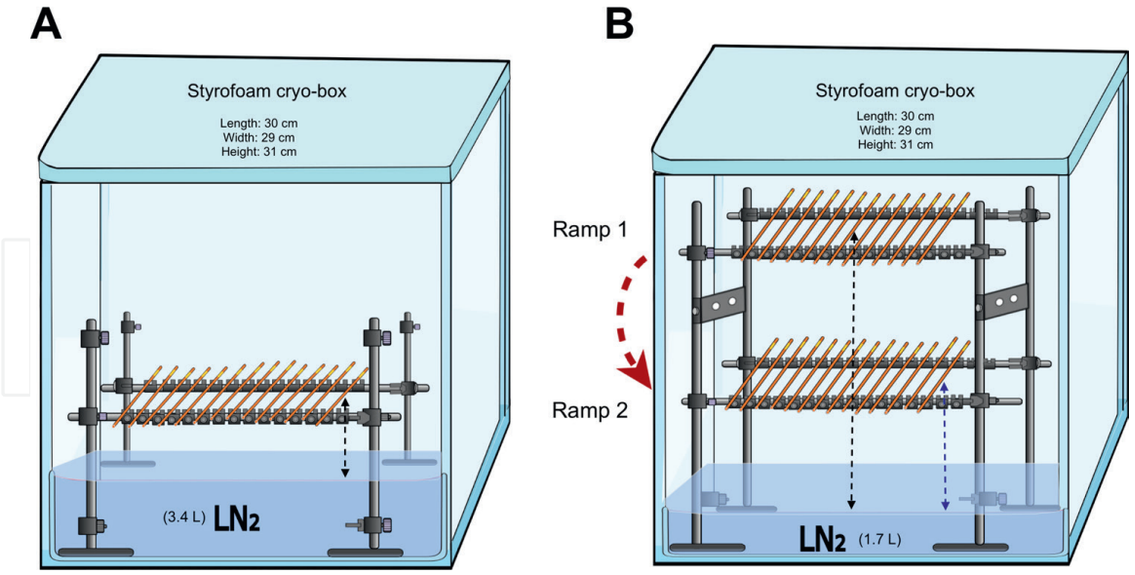

Figure 5.
Styrofoam cryo-boxes are used for cryopreserving domestic spermatozoa by conventional slow freezing. The conventional cryo-box has one freezing ramp located 4 cm from the LN_2 surface. The modified cryo-box has two freezing ramps located at either 7 and 17 cm or 10 and 24 cm from the LN_2 surface.

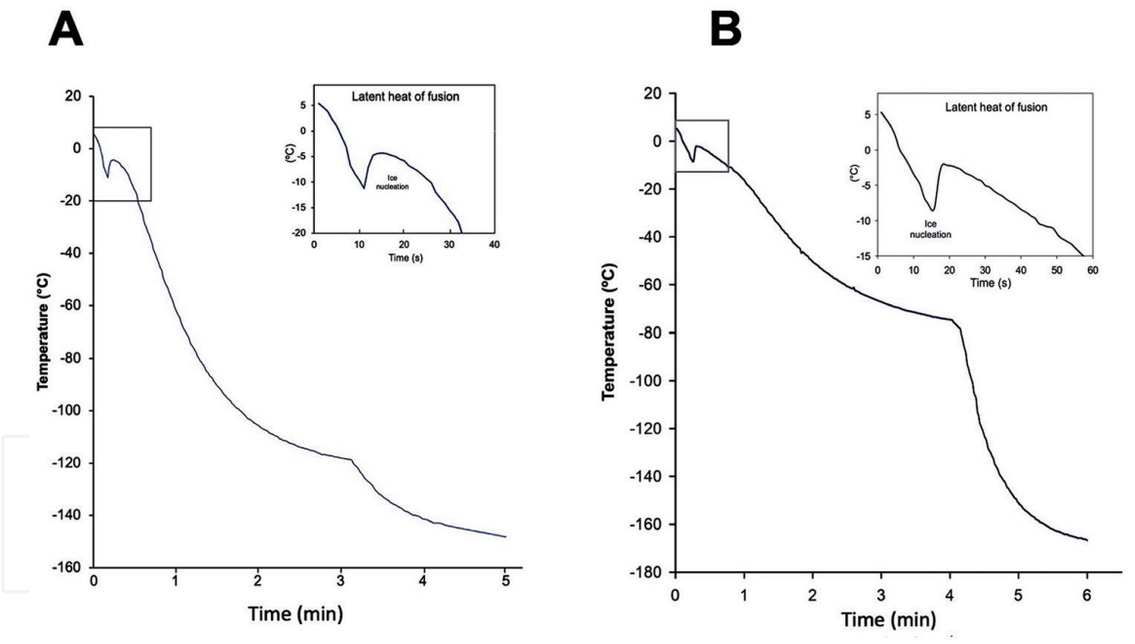

Figure 6.
Time courses of the temperature measured inside the straws (internal temperature) during the freezing process. The duration and temperature at which the latent heat of fusion (ice nucleation) occurs within the straws are shown in the small box. (A) Shows the freezing curve generated in epididymal Guinea pig spermatozoa, and (B) shows the freezing curve for Peruvian Paso horse spermatozoa [69].

the plasmatic and acrosomal membrane was obtained, constituting one of the first reports on the cryopreservation of guinea pig spermatozoa so far.

Another recent study used the same Styrofoam cryo-box with 3.4 liters of LN_2 to freeze Peruvian Paso horse spermatozoa [69]. This investigation used two ramps inside the cryo-box at two different heights: 17 and 7 cm above LN_2, to freeze the 0.25 ml straws, exposing them to LN_2 vapors for 4 and 2 minutes on each ramp,

respectively. Ice nucleation occurred at −8.5°C, and the dissipation of the latent heat of fusion lasted for 26 seconds. This freezing protocol generated accelerated cooling rates with an initial cooling rate of 9.2°C/min before and during ice nucleation, followed by an acceleration to 19 and 47°C/min (see **Figure 6B**). This protocol produced a plasma membrane integrity of 52.1 ± 2.19% and acrosomal membrane integrity of 87.6 ± 0.92%.

There are other cryopreservation procedures that involve extremely high cooling rates (~10,000-719,000°C/min). Among these procedures, "Ultra-rapid freezing" and "vitrification" have been defined. These cryopreservation methods use nonpenetrating CPA such as disaccharides (e.g., sucrose or trehalose) or trisaccharides (e.g., raffinose) sugars at high concentrations (100–500 mM), which can be toxic to cells. The disaccharides most commonly used in these protocols are sucrose and trehalose. During ultra-rapid freezing or vitrification, CPAs do not penetrate into the spermatozoa. Unlike vitrification of embryos or oocytes, experiences in vitrification of human spermatozoa by applying extremely high cooling rates (e.g., ~ 10,000–719,000°C/min) have allowed for the reduction or even elimination of the addition of permeable CPAs, thus avoiding the toxic effects on spermatozoa [70, 71]. This type of cryopreservation is known as "kinetic vitrification." However, this has led to controversies regarding the name of the technique, with some authors suggesting the use of "ultra-rapid freezing" instead of "kinetic vitrification." This technique does not prevent the formation of extracellular crystals, although they are smaller.

Kinetic vitrification and/or ultra-rapid freezing are carried out by direct immersion of the spermatozoa diluted in TCG-EY-100 mM sucrose in 30–50 μl drops directly into liquid nitrogen, which allows a cooling rate > 2000°C/min (**Figure 7**). In human spermatozoa, the application of classical vitrification with high concentrations of cryoprotectants has also resulted in extremely low or null sperm viability percentages. Alternatively, vitrification protocols have been designed based on the physical fact that extremely high cooling rates increase the viscosity of intracellular and extracellular fluids, which prevents all types of molecular diffusion and translates into a decrease or inability to form ice. In animals, kinetic vitrification of horse sperm, either in 30 μl spheres or in 0.25 ml straws (volume of 100 μl) using INRA-96 diluent (IMV Technologies, L'Aigle, France), 1% BSA, and 100 mM of trehalose resulted in greater sperm quality after warming than with conventional freezing using 5% glycerol. In addition, the effect of sucrose, trehalose, and raffinose was evaluated, and a better cryoresponse of equine spermatozoa with 100 mM trehalose was determined. In fact, the in vitro fertilizing ability of the vitrified-warmed equine spermatozoa reached the same fertility percentages as those obtained after conventional freezing [72–74].

The effectiveness of two methods for the cryopreservation of dog epididymal spermatozoa was evaluated. One method involved conventional slow freezing with reduced equilibration times (1 hour with 5% glycerol, then exposure of 2 min in LN_2 vapors after 2 hours at 5°C), while the other method involved ultra-rapid freezing with a nonpermeable cryoprotectant (250 mM sucrose). TCG-EY (20% egg yolk)-based extender was used in both cryopreservation methods. The results showed that the ultra-rapid freezing method produced a lower percentage of total and progressive motility and acrosome integrity compared to the conventional slow-freezing method. However, the kinetic variables and the integrity of the plasma membrane did not differ between the two cryopreservation methods. Furthermore, unlike the ultra-rapid freezing method, the width, area, and perimeter of the sperm head were reduced after the conventional freezing method. Therefore, these findings suggest that,

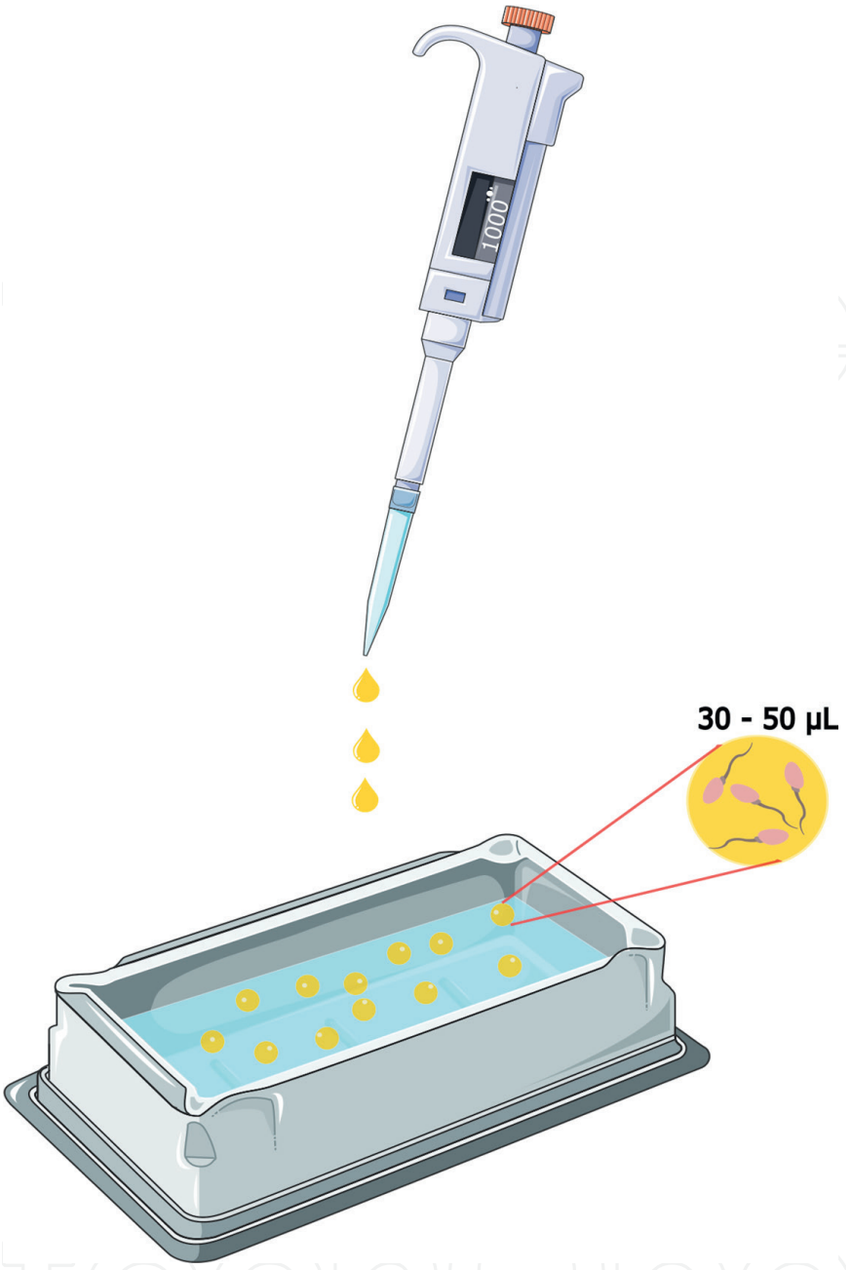

Figure 7.
Kinetic vitrification or ultra-rapid freezing of spermatozoa by direct immersion of 30–50 ul sperm samples into LN_2.

despite the low motility achieved after the ultra-rapid freezing method, the values of kinetics, viability, and morphometric dimensions of the head are similar to those obtained after conventional freezing. Thus, ultra-rapid freezing with sucrose may be a useful alternative to the cryopreservation of dog epididymal spermatozoa [75].

The effectiveness of two methods for the cryopreservation of dog epididymal spermatozoa was evaluated, one by conventional slow freezing with reduced equilibration times (from 2 hours at 5°C, which included 1 hour with 5% glycerol) and cooling (exposure of 2 min in LN_2 vapors), and the other by ultra-rapid freezing with nonpermeable cryoprotectant (250 mM sucrose). TCG-EY (20% egg yolk)-based extender was used in both cryopreservation methods. The results showed that the ultra-rapid freezing method produced a lower percentage of total and progressive

motility and acrosome integrity compared to the conventional slow freezing. However, the kinetic variables and the integrity of the plasma membrane did not differ between both cryopreservation methods. Furthermore, unlike the ultra-rapid freezing method, the width, area, and perimeter of the sperm head were reduced after the conventional freezing method. Therefore, these findings suggest that, despite the low motility achieved after the ultra-rapid freezing method, the values of kinetics, viability, and morphometric dimensions of the head are similar to those obtained after conventional freezing, ultra-rapid freezing. Rapid with sucrose may be a useful alternative to cryopreservation of dog epididymal spermatozoa [75].

The appropriate thawing rate for conventionally frozen sperm samples is 226°C/min, which can be achieved by immersing the straws or pellets in a 37°C water bath for 30 seconds. In some laboratories, good results have been obtained by thawing straws at 50°C for 9 seconds. In the case of ultra-rapid freezing or kinetic vitrification, it is recommended to thaw or warm the pellets between 63°C (60–65°C) for 1–2 seconds, using devices that allow immediate thawing or warming without overheating the sample (e.g., DDP-70® from INIA, Madrid, Spain or STC-3008 from Laboratory of animal reproduction biotechnology, University of Cuenca, Ecuador [**Figure 8**]) [76]. Another ultra-rapid thawing system is detailed by Galarza et al. [75], which adapts a flotation heating system by using sterilized glass beakers in a water bath at 65°C. Sperm pellets are placed in the glass beaker for 5 seconds, and then all liquid contents are immediately transferred to 1.5 ml Eppendorf tubes. Finally, the heated samples are centrifuged at 300 g for 5 minutes to remove sucrose (due to toxicity),

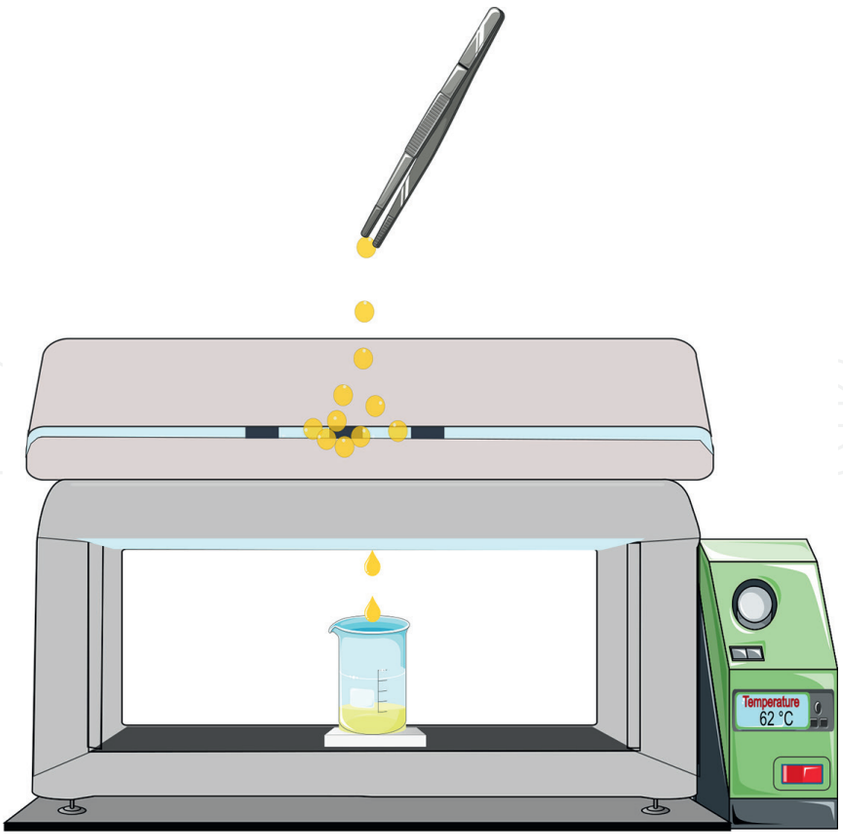

Figure 8.
Handmade warming device (STC-3008) containing thermal plates set at 63°C. Ultra-rapid frozen or vitrified spheres are exposed to these plates, and then the samples are rapidly dissolved in 1 to 2 seconds, with their contents received in a glass beaker.

and the pellet is resuspended in the same TCG-EY-based extender. Another warming method was described for vitrified horse samples; the spheres of straws were heated by being submerged into 2 ml of the INRA-96 extender at 42°C [72, 73].

In wild mammals, ultra-rapid freezing or kinetic vitrification techniques have shown variable results depending on the species. Notable results have been obtained in spermatozoa from dama gazelle (*Gazella dama*), brown bear (*Ursus arctos*), or giraffe (*Giraffa camelopardalis*) using a synthetic-based diluent (TCG: Tris, citric acid, and glucose), 6 to 20% EY, and 100 mM sucrose [14]. In ibex, successful demonstration of the fertilizing capacity of heated vitrified spermatozoa from both ejaculates and epididymis (*post-mortem*), has been achieved [77].

4. Cryoprotectant agents (CPAs)

Cryoprotectant agents (CPAs) are chemicals used in sperm cryopreservation to protect cells from damage that occurs during the freezing and thawing process. During freezing, the water inside and outside the cells expands and forms ice crystals, which can damage the cells and reduce their fertilizing ability. CPAs protect cells by decreasing the formation of ice crystals and preventing cell dehydration. To ensure the cryosurvival of sperm, it is necessary to optimize an adequate cooling rate that allows for the alteration of the physicochemical behavior of the aqueous solutions in which cryopreservation occurs. For this reason, CPAs are added to the cryopreservation medium, which mainly reduces the eutectic point of the solution (the minimum temperature at which the solution remains in a liquid state). Cryoprotectant agents (CPAs) are necessary to modify the rate of water transport, ice nucleation, and ice crystal formation [78]. There are two types of CPAs: 1) penetrating CPAs (such as GLY, dimethyl sulfoxide [DMSO], dimethylacetamide [DMA], dimethylformamide [DMF], 1,2 propandiol, ethylene glycol [ETG], propylene glycol, among others), which are characterized by their low molecular weight and their ability to optimally dehydrate the cell at low cooling rates; and 2) nonpenetrating CPAs (such as polyvinylpyrrolidone [PVP], glucose, fructose, sucrose, raffinose, trehalose, among others), which have a high molecular weight and are more effective when used at high cooling rates [77]. The molecule size of nonpenetrating CPAs is larger than that of penetrating CPAs such as glycerol. Nonpenetrating CPAs do not penetrate the cell but rather exert their cryoprotective action by favoring rapid cell dehydration and promoting viscosity, thus decreasing the formation of ice crystals. Nonpermeable CPAs are also often used in association with permeable CPAs. These components contribute to the extraction of intracellular water of different osmotic pressures without penetrating the cell. In most cases, the freezing medium usually contains both CPAs [79].

GLY is the main penetrating CPA used to freeze mammalian sperm [80], but some effects remain unclear. Early studies reported that GLY reduces the quality of fresh and frozen-thawed semen in some species [81]. It has also been reported to be harmful when added to diluents above 30°C, as well as at high concentrations greater than (> 6%) or less than 2% [82]. GLY is generally used at concentrations of 0.5–1.5 M (approximately 4–10% v/v) [83]. Indeed, elevated GLY concentrations have a negative effect on sperm membrane integrity [84]. Furthermore, GLY is osmotically active, changing the water content of sperm [85]; any induced osmotic stress could reduce sperm longevity and accelerate sperm capacitation. Only a few studies have been conducted examining the addition of GLY to extenders for cooling [86]; therefore, their effects on sperm functionality, especially kinematic variables and

mitochondrial integrity, need to be further investigated. Previous studies have evaluated the in vitro effect of 5% GLY, 3% or 5% ethylene glycol, or 3–5% acetamide on frozen-thawed ram spermatozoa and have determined that 5% GLY or 3–5% ethylene glycol protects ram sperm against the harmful effects of freezing and that GLY at 5% offers greater protection to the sperm plasma membrane [87].

Another point of interest is the exposure time of sperm to the CPA before the cryopreserving process. This time is commonly referred to as the "equilibration time." This period must allow for proper stabilization of the sperm membrane and varies depending on the species and the origin of the sperm (ejaculate or epididymal). Unlike epididymal spermatozoa, ejaculated spermatozoa require longer equilibration times, which vary significantly depending on the species, ranging from 2 to 4 hours. In a study carried out on ejaculated semen from bulls native to the Ecuadorian Andes, the sperm were equilibrated for 2, 4, and 7 hours prior to freezing in two types of commercial extenders (Andromed® and Triladyl®). It was determined that there were no significant differences between times or extenders [88]. On the other hand, in a study carried out on dog epididymal spermatozoa, the sperm were exposed to 5% GLY for only 1 hour to equilibrate the membranes. They were then subjected to freezing in LN_2 vapors using a slow-freezing protocol with two ramps for 2 min (1 min in each ramp). This protocol, shortened the equilibration time of dog epididymal spermatozoa, allowing for total motility of 37.7 ± 4.43% and integrity of the plasmatic and acrosomal membranes of 39.7 ± 1.88%, making it a fast and attractive protocol.

A study carried out on Arabian horse sperm showed that the addition of 5% DMF or a combination of 3% GLY and 3% DMF (amide) to the freezing medium (Botusemen Gold®) resulted in higher kinematic parameters and integrity of plasma and acrosome membranes compared to using 5% GLY alone, regardless of the freezing method (by acceleration or deceleration) [89, 90]. However, the in vitro fertilization ability (assessed by heterologous IVF) of those Arabian horse spermatozoa previously frozen with DMF-GLY (3–3%) was similar to that obtained after using 5% GLY alone or 5% DMF alone [91].

Conventional slow freezing and ultra-rapid freezing have been developed for epididymal or ejaculated spermatozoa from wild mammals using sucrose (as a nonpenetrating CPA) and GLY (as a penetrating CPA). An extender based on TCG plus EY (at 6%, 12%, or 20%) with 5% GLY (osmolarity: 1150–1164 mOsm/kg) and 100 mM sucrose (osmolarity: 442–486 mOsm/kg) has been used for freezing or ultra-rapid freezing of sperm from Aoudad (*Ammotragus lervia sahariensis*), Iberian ibex (*Capra pyrenaica*), Chamois (*Rupicapra pyrenaica*), European wisent (*Bison bonasus),* Dama gazelle (*Gazella dama*), Saharawi dorcas gazelle (*Gazella dorcas neglecta*), Sitatunga (*Tragelaphus spekii*), Giraffe (*Giraffa camelopardalis rothschildi*), Pere David's deer (*Elaphurus davidianus*), Fallow deer (*Dama dama*), Muntjac (*Muntiacus muntjak*), Barbary macaque (*Macaca sylvanus*), and Bottlenose dolphin (*Tursiops truncatus truncatus)*. Likewise, an extender based on TEST plus 12% EY with 4 to 6% GLY (osmolarity: 859–1245 mOsm/kg) or 100 mM sucrose (osmolarity: 430 mOsm/kg) was used to cryopreserve spermatozoa of Brown bears (*Ursus arctos*), Giant pandas (*Ailuropoda melanoleuca*), and Queensland koalas (*Phascolarctos cinereus adustus*). In these wild species, the cryoresistance ratio (CR) of their motility and viability was calculated using the formula: (*value after thawing/value before thawing) x 100*. For the fallow deer, mouflon, chamois, sitatunga, dorcas gazelle, dama gazelle, giraffe, and brown bear, ultra-rapid freezing produced good results (around 40% or higher) for CR-motility and CR-viability values from epididymal spermatozoa. Nevertheless, the sitatunga samples returned the worst results. In fact, ultra-rapid freezing appeared to be lethal to this species' sperm. The CR-viability values

recorded for the ultra-rapidly frozen Iberian ibex, mouflon, Pere David's deer, and giant panda sperm were reasonably good (around 40%). In addition, the morphometric head dimensions (area and length) of epididymal or ejaculate spermatozoa from those wild species varied after conventional freezing or ultra-rapid freezing compared with fresh values. The area of sperm head of dolphins and giraffes increased after conventional freezing-thawing. In the chamois, Iberian red deer, sitatunga, and dorcas gazelle, they did not change. In all the remaining wild species, the head area decreased after both conventional and ultra-rapid freezing. In summary, ultra-rapid freezing is especially recommended for the cryopreservation of epididymal sperm from the dama gazelle, giraffe, and brown bear. For the other species, slow freezing appears to provide better results with both epididymal and ejaculated sperm [14].

In Ecuador, research has been carried out on white-tailed deer (*Odocoileus virginianus*) and chorongo monkeys (*Lagothrix poeppiggi*). In these wild species, the kinematic variables, integrity of sperm membranes, and morphometric dimensions were evaluated from samples of both fresh and conventionally frozen or ultra-rapidly frozen sperm.

White-tailed deer spermatozoa had better cryoresistance to conventional freezing than to ultra-rapid freezing. In fact, ultra-rapid freezing reduces the dimensions of the head (**Table 1**). On the other hand, Chorongo monkey spermatozoa were drastically affected by both freezing types. Contrary to deer, ultra-rapid freezing increased the head dimensions of the monkey's spermatozoa (**Table 2**).

Morphometric sperm dimensions are depicted in **Figure 9**.

Variables	Fresh (n = 12)	Post slow freezing (n = 12)	Post ultra-rapid freezing (n = 12)
Kinematic variables			
Total motility (%)	72.4 ± 1.13^{a}	63.4 ± 3.81^{a}	26.6 ± 4.40^{c}
Progressive motility (%)	32.6 ± 3.56^{a}	15.3 ± 2.20^{b}	6.7 ± 1.46^{b}
VCL (μm/s)	78.3 ± 5.01^{a}	44.2 ± 3.50^{b}	48.0 ± 6.66^{b}
VSL (μm/s)	34.7 ± 3.11^{a}	14.9 ± 1.26^{b}	17.3 ± 2.60^{b}
ALH (μm)	2.90 ± 0.32	2.13 ± 0.14	2.62 ± 0.31
BCF (Hz)	7.42 ± 0.27^{a}	5.87 ± 0.34^{ab}	3.34 ± 0.48^{b}
Integrity of membranes			
Total IP (%)	75.8 ± 3.22^{a}	59.1 ± 1.81^{b}	23.8 ± 0.88^{c}
Total IA (%)	91.3 ± 0.85^{a}	67.0 ± 1.68^{b}	43.2 ± 1.85^{c}
Sperm head dimension			
Length (μm)	7.26 ± 0.10^{a}	7.35 ± 0.04^{a}	6.24 ± 0.05^{b}
Width (μm)	4.28 ± 0.06^{a}	4.47 ± 0.04^{a}	3.72 ± 0.15^{b}
Area (μm^{2})	29.75 ± 0.77^{a}	29.65 ± 0.29^{a}	20.74 ± 0.17^{b}
Perimeter (μm)	16.63 ± 0.20^{b}	17.73 ± 0.13^{a}	14.72 ± 0.13^{c}

Different superscripts in each row express significant differences $^{a-b-c}$ $P < 0.05$; $^{a-c}$ $P < 0.01$.

Table 1.
*Variables of epididymal spermatozoa from six White-tailed deer (**Odocoileus virginianus**) either fresh, conventionally frozen, or ultra-rapidly frozen.*

Variables	Fresh (n = 12)	Post slow freezing (n = 12)	Post ultra-rapid freezing (n = 12)
Kinematic variables			
Total motility (%)	67.3 ± 12.1[a]	11.0 ± 2.00[c]	11.8 ± 3.33[c]
Progressive motility (%)	29.9 ± 15.0[a]	2.2 ± 0.60[c]	2.3 ± 0.20[c]
VCL (μm/s)	51.1 ± 11.5[a]	32.9 ± 3.50[b]	31.2 ± 3.60[b]
VSL (μm/s)	24.9 ± 8.29[a]	17.8 ± 2.90[b]	12.8 ± 1.13[b]
ALH (μm)	2.39 ± 0.30[a]	1.69 ± 0.12[b]	1.60 ± 0.30[b]
BCF (Hz)	6.43 ± 1.72[a]	6.32 ± 1.02[a]	3.24 ± 0.31[b]
Integrity of membranes			
Total IP (%)	80.4 ± 5.97[a]	12.1 ± 1.91[c]	12.0 ± 1.38[c]
Total IA (%)	94.4 ± 0.32[a]	75.9 ± 1.48[b]	65.3 ± 2.42[c]
Sperm head dimension			
Length (μm)	4.98 ± 0.04[b]	4.78 ± 0.05[b]	5.14 ± 0.05[a]
Width (μm)	2.87 ± 0.03[b]	2.81 ± 0.03 [b]	2.98 ± 0.02[a]
Area (μm^2)	14.32 ± 0.33[b]	14.81 ± 0.25[b]	16.3 ± 0.28[a]
Perimeter (μm)	10.95 ± 0.13[b]	10.99 ± 0.12[b]	11.85 ± 0.12[a]

Different superscripts in each row express significant differences [a – b – c] $P < 0.05$; [a – c] $P < 0.01$.

Table 2.
*Variables of epididymal spermatozoa from two Chorongo monkey (**Lagothrix poeppiggi**) either fresh or freezing conventionally or ultra-rapid.*

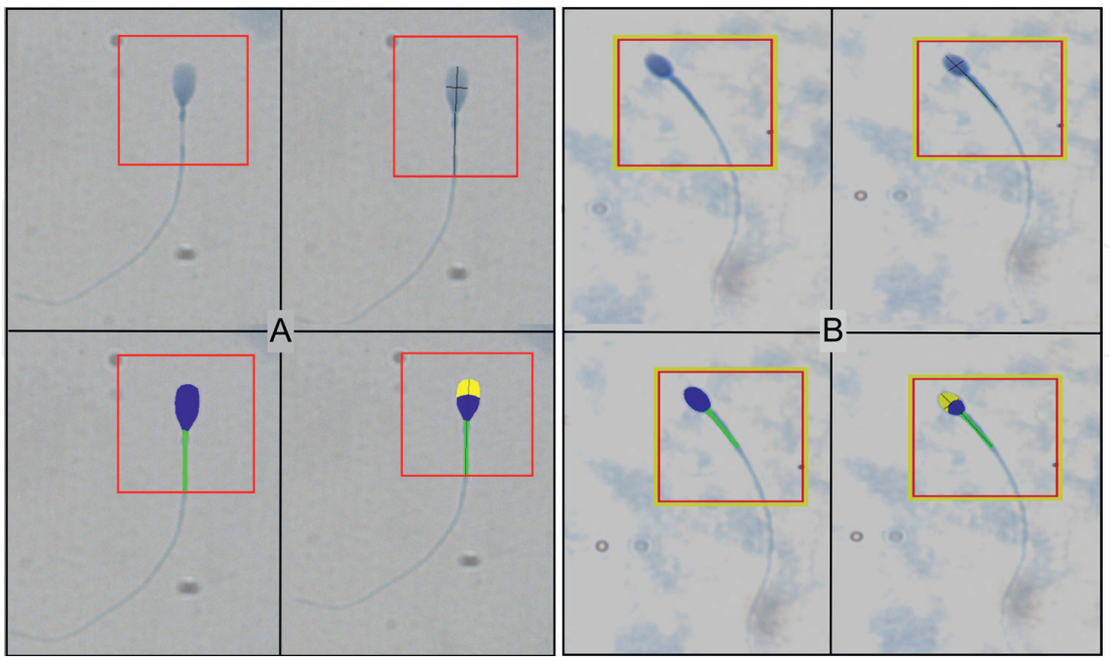

Figure 9.
Sequential measurement of the dimensions of the head and midpiece of White-tailed deer (A) and Chorongo monkey (B) spermatozoa recorded by the ASMA (automated sperm morphometry analysis) module of the CASA system (sperm class Analyzer, SCA-evolution® 2018, v.6.4.0.99 software; Microptic S.L., Barcelona, Spain).

5. Extenders and additives

Nowadays, there are several extenders available for commercial use that include a buffer component (salts), an acid, and a sugar source in their basic composition. Most diluents also have additives such as antioxidants and cryoprotective agents (CPAs) to protect sperm against cold shock during the cooling, freezing, and thawing processes. However, both commercial and laboratory-made diluents must meet important pH and osmolarity requirements: close to neutrality (pH 6.8 to 7.2) and between 280 to 320 mOsm/kg. In general, extenders must be hypertonic or isotonic rather than hypotonic to prevent an osmotic difference and the disintegration of spermatozoa structure. The most commonly used buffer components worldwide are Tes, Tris, Hepes, Pipes, Mes, Tricine, and citric acid, which are organic substances that act as external cryoprotectants and protect cells against cold shock that occurs during cooling from 30–5°C. Other components include EY, skimmed milk, soy protein, which also act as cryoprotectants, as well as sugar sources like glucose and fructose, and antibiotics such as penicillin and streptomycin.

Extenders with egg yolk (EY) are commonly used to cryopreserve livestock sperm, such as that cattle, buffalo, pigs, and dogs. Although EY is known to prevent cell damage during cryopreservation, the presence of substances in yolk granules, including high-density lipoproteins (HDL) and minerals, inhibits the respiration of sperm cells and reduces their motility. However, the low-density lipoproteins (LDL) of EY protect sperm from damage by covering the sperm membrane during freezing and thawing. Although most extenders include EY, alone, some are supplemented with GLY, and there are concerns over biosecurity and the possibility that egg contents might alter sperm structure and physiology [92]. Despite those drawbacks, EY represents one of the additives that provide the greatest benefits for sperm cryopreservation since it protects the cell from cold shock and provides some protection during the chilling-warming, freezing-thawing, or vitrifying-warming processes. EY prevents the loss of cholesterol and phospholipids from the sperm membrane by absorbing seminal plasma proteins, decreasing their availability to bind to the membrane, and, therefore, dampening their deleterious effect on the output of cholesterol and phospholipids [93]. In addition, EY lipoproteins bind to the plasma membrane, providing protection against cold shock during cooling and freezing. Researchers have added 6% EY to the TEST extender (Tris, Tes, and glucose) plus 5% GLY to freeze ram semen, obtaining good cryosurvival results (motility: 61.4 ± 1.9% of motility and viability, and 2.3 ± 0.5% of DNA fragmentation) [27]. Likewise, ram semen diluted with TEST +6% egg yolk +5% GLY and cold-stored for 24 hours showed that the EY protected sperm against cold shock and allowed the sperm membrane to be maintained after 24 hours of refrigeration, irrespective of the sperm selection method (density gradient centrifugation or filtration) [93].

On the other hand, adding 6% EY (v/v) to a UHT (ultra-heat-treated 'long-life') skimmed milk-based extender plus antibiotics has been shown to be as effective as the TEST diluent in preserving the motility and integrity of ram spermatozoa membranes for 96 hours at 5°C. In addition, the effect of sperm filtration with Sephadex G-15® columns and the addition of 2% GLY was evaluated. However, despite having good motility, kinetics, and integrity of sperm membranes during storage, it was determined that the UHT extender with or without 2% GLY produced better sperm quality results than the TEST extender with or without GLY for storage at 5°C for 96 hours. It was even determined that ram semen stored for 48 hours in the UHT diluent +6% EY had the same *in vitro* fertility as fresh semen [94–96].

Antioxidants and additives can ameliorate the detrimental effects of ROS increase and improve the quality of cryopreserved sperm, whether chilled-warmed or frozen-thawed. Several enzymatic antioxidants, for example, reduced glutathione (GSH), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT), protect against LPO formation in frozen and thawed mammalian sperm, allowing acceptable motility values and percentages of cells showing the integrity of the plasma membrane [92, 93]. Other new additives-antioxidants such as resveratrol, vitamin E and their combination, taurine, trehalose, quercetin, palmitoleate, butylated hydroxytoluene, and kinetin have been shown to help maintain motility variables and the integrity of sperm membranes (plasma and acrosome) during storage at 4–5°C [97–104].

L-*carnitine* (LC) is an additive synthesized from the two essential amino acids lysine and methionine. It plays an important role in the transport of short-, medium-, and long-chain fatty acids into mitochondria for β-oxidation and increased ATP production, thus having a positive effect on motility, maturation, and the entire spermatogenic process of sperm. LC's antioxidant properties also help protect against ROS. In vitro studies have shown that carnitines improve human sperm motility and may have a cryoprotective effect [105–107]. The effects of supplementing extenders with LC have been examined in bulls, buffalo, goats, and rabbit sperm, with some beneficial effects observed. A recent study reported that adding LC to a synthetic-based extender for frozen and thawed ram semen did not have consistent benefits. Another study determined that the most suitable dose of LC supplemented with UHT-EY (6%) extender was 5 mM, which produced the greatest motility, kinetics, and membrane integrity results. Additionally, it demonstrated that 5 mM LC improves the in vivo fertilizing capacity of ewes when the ram semen is diluted with a UHT extender plus 5 mM LC [108–113].

Likewise, it has been shown that 25 mM LC (vs. 0, 1, 5, 10, and 50 mM of LC) added to the INRA-96 and UHT diluents was the most suitable dose to preserve horse semen during 96 hours of storage at 5°C. Subsequently, 25 mM was added to the commercial 'INRA-Freeze®' freezing medium, and it was determined that LC did not improve the kinetic or progressivity variables; however, it protected the plasmatic and acrosomal membranes with respect to its control, thus determining a cryoprotective effect on the sperm of the Peruvian Paso horse [114]. L-carnitine has also been used for freezing or vitrifying dog epididymal spermatozoa. A study demonstrated that 5 mM LC supplementation to freezing (TCG + 20% EY + 5% GLY) or vitrifying (TCG + 20% EY + 250 mM sucrose) media produced desirable results in kinematic parameters, the integrity of membranes, and fertilizing ability. The results showed that the integrity of plasma/acrosome membranes was greater in sperm frozen or vitrified with L-carnitine than in the control group (nonsupplemented samples). Moreover, dog epididymal sperm freezing with L-carnitine yielded a higher proportion of pronuclei formation than sperm vitrification without LC (69.2 ± 2.46% vs. 61.0 ± 1.51%). Additionally, significant correlations between the kinematic parameters (TM, PSM, VCL, and VSL) and pronuclear formation were found in samples freezing or vitrifying with LC. Therefore, the authors suggested that the addition of LC to the freezing and vitrification media produces a positive cryoprotective effect on the plasmatic and acrosomal membranes, even improving the fertilizing ability of dog epididymal spermatozoa [115].

Similarly, it has been shown that melatonin (ME) is a powerful free radical scavenger with the ability to repair damaged biomolecules in the cells of living organisms. Due to its amphiphilic nature, ME can cross cell membranes and easily reach intracellular

compartments, including mitochondria. Additionally, caffeine (CAF), a methylxanthine-derived phosphodiesterase inhibitor, has been proven to improve sperm motility. CAF binds to adenosine receptors and stimulates adenylyl cyclase, an enzyme present in mitochondria that facilitates the conversion of ATP to cAMP [116–118]. The skimmed milk-based extender was added with concentrations of 1 μM (ME) and 2 mM (CAF) to the freezing medium, and it was determined that they protect the plasmatic and acrosomal membranes during the cryopreservation process. Nevertheless, neither CAF nor ME improved the oxidative stress after the cryopreservation process [69].

In wild species such as the Iberian ibex, replacing EY with other additives such as lactose produced a significant decrease in sperm motility after thawing. The use of extenders containing low concentrations of EY (e.g., 6%) has been recommended. In mouflon epididymal spermatozoa, the integrity of the plasma membrane and the different sperm kinetic variables are better preserved with an EY percentage of 6% than 12%. When extenders based on EY are used, the seminal plasma of the Iberian ibex exerts a deleterious effect on the preservation of spermatic material due to the presence of a phospholipase of bulbourethral origin. There is an interaction between the equilibration time with the cryoprotectant and the type of extender, in different parameters of post-thawing sperm quality. The best results were obtained in terms of greater motility, viability, and integrity of the acrosome in semen obtained from electro-ejaculation when using the TEST-EY diluent and an equilibration time of 3 h at 5°C, compared to the 2 h classically used in this species. In the case of the heron (*Ammotragus lervia*), as indicated in mouflon and other ovine species, the use of the zwitterionic agent Tes (TEST extenders) determines a favorable effect on sperm cryopreservation [119–122].

Cryopreservation of Spix's yellow-toothed cavy (*Galea spixii Wagler, 1831*) epididymal sperm using Tris- and coconut water (ACP®-116c)-based extenders supplemented with 10% or 20% egg yolk or Aloe vera suggested that the use of Tris plus 10% egg yolk was the most effective in preserving sperm kinetic parameters (68.1 ± 5.9% motile sperm) and membrane integrity (48.2 ± 7.4%) [123]. Cryopreservation of the semen of a capuchin monkey (*Cebus apella*) demonstrated that the TES–TRIS (3.5% glycerol) extender was more appropriate than the coconut water solution (CWS) plus 2.5% glycerol). However, CWS + caffeine potentially increases sperm motility and may be useful in the AI of freshly diluted semen [124].

Resveratrol (RES) (3,5,40-trihydroxystilbene) is a polyphenolic antioxidant compound consisting of two aromatic rings connected by a methyl bridge (2,2-azinobis; 3-ethylbenzothiazoline-6-sulfonic; 1,1-diphenyl-2-picrylhydrazyl) that has been used as an antioxidant in cryopreserved spermatozoa. RES is a potent natural additive present in different dietary principles such as grapes, red wine, berries, pistachios, plums, and peanuts [125]. It has a similar structure to diethylstilbestrol and estradiol [126]. The antioxidant effects of RES allow for a decrease in LPO by acting as a direct scavenger of ROS during the freezing and thawing of spermatozoa. Additionally, it indirectly acts as a chelating agent for copper and iron, reducing apoptosis in germ cells and increasing spermatogenesis by stimulating the hypothalamic-pituitary-gonadal axis without generating any side effects [127].

Supplementation of RES to freezing media has increased bull sperm quality, improving motility, mitochondrial activity, and DNA integrity. Assunção et al. [128] evaluated the most suitable RES dose (0.05, 0.1, and 1 mM) supplemented with the freezing medium and showed that the concentration of 0.05 mM better protected the integrity of the plasma and acrosomal membranes. However, its effect was not evident *in vitro* fertility. Similarly, Li et al. [129] demonstrated that RES significantly reduces

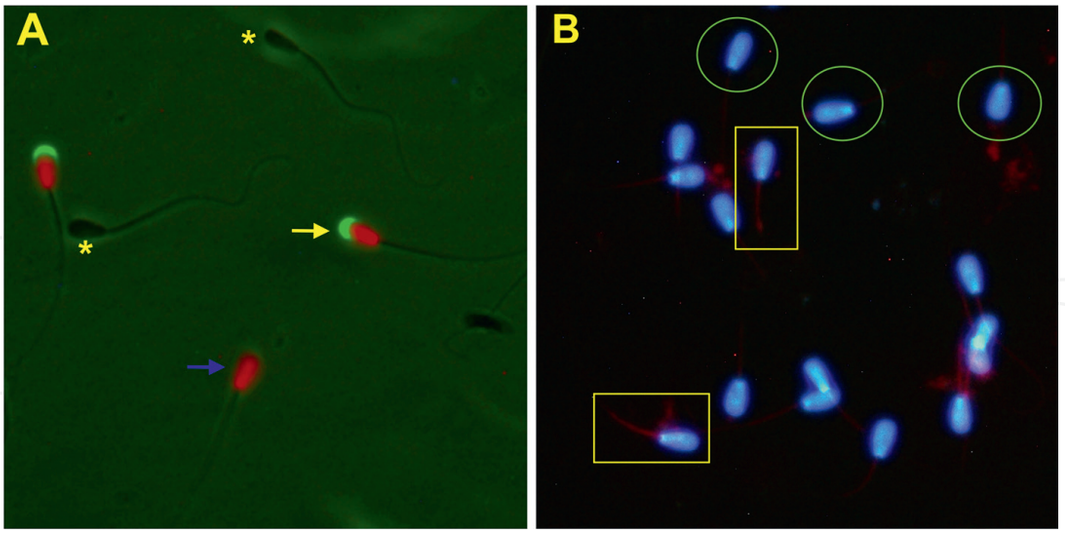

Figure 10.
Bovine spermatozoa observed under an epifluorescence microscope (Nikon eclipse ci-E, negative phase contrast [Ph1] with a green filter; Nikon instruments, Inc., New York) with a triple-bandpass filter (B-2A, excitation: 450–490 nm, emission: 520 nm; G-2A, excitation: 510–560 nm, emission: 590 nm; and UV-2E/C, excitation: 330–380 nm, emission: 420 nm). (A) Spermatozoa stained with PI/PNA-FITC double fluorescent test. notice the intact spermatozoa (asterisks, nonstained), spermatozoa with damaged plasma membrane (blue arrow), and spermatozoa with damaged plasma membrane and acrosome. (B) Spermatozoa stained with the CellROX deep red reagent® fluorescent probe (2.5 mM; CAT 10422 life technologies) and Hoechst 33342. Notice the spermatozoa without oxidative stress (green circles) and spermatozoa with oxidative stress based on the red staining of the midpiece (yellow rectangles).

harmful levels of ROS, protects mitochondrial function and acrosomal integrity, and, unlike Assunção et al. [128], increases *in vitro* fertility. In buffaloes, the addition of RES to the TCG-extender improved sperm quality parameters, ATP levels, sperm fertilizing capacity, and efficiently decreased DNA fragmentation and LPO during cryopreservation [130]. In small ruminants, RES reduced oxidative stress and increased fertility and longevity after the freezing process [131]. In boars, RES increased *in vitro* fertilizing capacity [132] and reduced induced oxidative stress [126]. In rabbits, RES improved fertility by enhancing androgens by acting directly on estrogen receptors, allowing an increase in the number and motility of epididymal spermatozoa [133].

A recent research conducted in our laboratory evaluated the antioxidant and cryoprotective effect of RES supplemented with TCG-EY medium for freezing and vitrifying bovine sperm. The results showed that RES improved the integrity of the plasma and acrosomal membranes before freezing. Furthermore, the cryoprotective effect of RES was evidenced after the freeze-thaw process, improving total and progressive motilities as well as the integrity of plasma and acrosomal membranes. On the other hand, RES improved the values of beat-cross frequency (BCF) and decreased oxidative stress in samples subjected to ultra-rapid freezing (**Figure 10**). The results suggest that RES provided an antioxidant and cryoprotective effect, improving kinematics, protecting membrane integrity, and decreasing oxidative stress during conventional or ultra-rapid freezing.

6. Conclusions

All efforts developed for optimizing sperm cryopreservation in domestic and wild animals continue remains a great challenge due to low cell cryosurvival rates

and, indeed, low fertility rates. The new approaches that must be taken into account for the current cryopreservation protocols must be aimed at improving the freezing protocols with accelerating cooling rates. The initial cooling rates must be low during the critical temperature range where ice formation occurs. The interaction of diluents, penetrating and nonpenetrating CPAs, and additives that mitigate the deleterious effects of cryopreservation may be an alternative to achieve greater cell cryosurvival. In species where the individual factor is very marked, it is recommended to try new protocols that are adjusted to obtain a better sperm cryoresistance response. Finally, to improve the protocols of ultra-rapid freezing and kinetic vitrification that conjugate disaccharides or the combination between permeable and nonpermeable CPAs to achieve desirable cell survival rates as has been determined in the human species.

Acknowledgements

The authors would like to thank the coordinators of the Yurak-Allpa Zoo-Refuge (Cuenca, Ecuador) Mr. Alberto Vele and MVZ(C). Wilson Cevallos, for their support in obtaining samples from wild animals.

Conflict of interest

The authors declare no conflict of interest.

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