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**REVIEW ARTICLE**

# Cryopreservation of Stallion Semen: A Review

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## Abstract

The cryopreservation of domestic animal sperms is a complicated process that necessitates the control of numerous factors in order to get the desired outcomes. Appropriate diluents and sperm dilution , cooling , and thawing rates are required to guarantee even a minimum success. To maximize the post-thaw recovery of sperm and, consequently, fertility, thorough knowledge of the intricate physiology of sperm for each species is also necessary. Cryopreserved semen is far less frequently utilized in equine artificial insemination (AI) than fresh or cooled-stored semen. This is caused, at least in part, by variations in the ability of different stallions' frozen-thawed semen to fertilize. Cryopreservation of semen is also connected with high prices. The breeding of animals has benefited greatly from the use of cryopreserved semen for artificial insemination during the past seven decades. Mammalian sperm were among the first cells to be effectively cryopreserved. The economic effectiveness of commercial artificial insemination using cryopreserved semen is therefore influenced by the number of insemination doses obtained per ejaculate, and also the one of the most challenging materials in the industry of equine semen is cryopreserving effectively without apoptosis or membrane damage.Stallions differ in their spermatozoa's cryotolerance; however, it is still unclear how much this variation is influenced by breed. Common cryopreservation methods for spermatozoa include direct submersion in liquid nitrogen and cryoprotectant-free vitrification. However, there is a chance that microbial contamination could occur with the direct freezing of sperm in commercial liquid nitrogen.So, cryopreservation aimed to protect and elongated the viability of sperms. Low-molecular-weight compounds that are very permeable are called cryoprotectants. By lowering the freezing temperature of the samples, spermatozoa are protected from freeze damage by ice crystallization. Glycerol, ethylene glycol, dimethyl sulfoxide (DMSO), and 1,2-propanediol are the four cryoprotectants that are most frequently utilaized. One of the most challenging materials in the industry of equine semen is to cryopreserve effectively without apoptosis or membrane damage. Therefore, this review explains in detail the steps of cryopreservation, its importance, and the types of cryoprotectants which added to stallion semen.

**Keywords:**
Cryopreservation, Stallion, Extenders,Frozen semen,(INRA 196), Glycerol,Amides.

## Introduction

The consecutive steps of temperature decrease, cellular dehydration, freezing, and thawing make up the cryopreservation process. The simplest technique and the fundamental idea

underlying the cryopreservation procedure, which successfully lowers the sperm's metabolic rate and lengthens its survival, is cooling spermatozoa. The most preferred and often utilized method for maintaining spermatozoa's capacity to fertilize is freezing semen at 196°C [1]. The semen straws are commonly preserved at 196°C using a variety of diluent solutions, including glycerol-egg yolk-citrate, milk glycerol, and glycerolated-egg yolkhydroxymethylaminomethane(TRIS).

Two important cryopreservation methods for preserving the semen are conventional slow freezing or programmed freezing and vitrification [2]. The most popular method is slow freezing, which cools spermatozoa at a rate of 1-2°C/min in increments up to 196°C. The disadvantages of this conventional freezing technique are primarily caused by heterogeneous ice nucleation, or the uncontrolled growth of ice crystals that disrupt sperm cells, and secondarily by insufficient latent heat dissipation, which is caused by repeated freeze-thaw cycles that mechanically damage cells [3]. Intracellular ice crystal disrupts cytoskeleton, whereas the extracellular ice crystal increases solute concentration and increases osmolarity, triggering loss of water from cells [4, 5]. A modest rate of cooling followed by four hours of equilibration and freezing is said to be a better cryopreservation strategy for Haryana bull semen since cooling rates have a significant impact on different seminal characteristics [6]. For spermatozoa to adapt to low temperatures and to permit the transfer of water, the equilibration period is crucial. This reduces the damage caused by ice nucleation during freezing-thawing[7, 8].

Sperm can be preserved by being kept in liquid nitrogen at a temperature of -196 °C. This prolongs the life of cells [9]. There are several crucial steps involved in the cryopreservation and usage of frozen semen. The stallion's semen is first collected, and the gel portion is then filtered out. Semen is promptly assessed, and the initial semen extension is carried out using an extender based on skim milk. Once the sperm pellet has been extended, the seminal plasma is removed, and it is then resuspended in an extender designed for freezing. In the cryopreservation procedure, seminal plasma removal is a crucial step to prevent harm to the quality and lifespan of preserved semen for extended periods of time [10] and can be performed by centrifugation or filtration [11]. The semen must first be cooled from room temperature (about 20°C) torefrigerator temperature (5°C)before the freezing process has begun. The cellular plasma membrane transitions from a liquid crystalline to a gel form at these temperatures, making it more vulnerable to damage from thermal stress. The semen also loses its ability to move and become fertile as a result. However, by regulating the cooling rate and including cryoprotectants in the semen extender, this effect can be reduced. The optimal cooling rate should be quick enough to prevent prolonged exposure of sperm to hyper-saturated solutions from the extracellular environment while slow enough to allow for adequate cell dehydration and prevent the production of intracellular ice crystals [12, 13]. Benefits of sperm cryopreservation include optimizing stallion use and producing more offspring with high genetic merit. It permits the preservation of genetic material for an infinite amount of time, lowers transportation costs and hazards, reduces geographic barriers, decreases the spread of illness, and allows the use of stallions that are physically incapable of

mating or have already passed away [14], and also promotes the preservation of the genetics of outstanding stallions as well as the international trade in semen[15]. Reliable freezing procedures will be required as soon as sperm sex sorting is implemented in order to optimize the usage of sex-sorted sperm in the equestrian sector[16, 17]. One of the most important tasks at semen cryopreservation was to select cryo-diluents that not only increased semen volume but also protected spermatozoa at the time of dilution, cooling, and freezing-thawing [18]. Variation in the effectiveness of diluents in preserving the motility and functional integrity of sperm during cryopreservation can be attributed to their different constituents.

**1. Steps of cryopreservation**:

A safe freeze-thaw technique is still lacking despite the adoption of several cryoprotectants and a standard, ideal freezing and thawing pace. To ensure an adequate supply of viable spermatozoa, it is best to count them in each sample prior to insemination of the mare [19]. Five steps can be used to organize the handling and preparation of semen for cryopreservation.(Figure 1)

***1.a. Removal of seminal plasma***

It was achieved to collect spermatozoa,because it has been demonstrated that seminal plasma can harm spermatozoa on its own before chilling and being stored at 5 °C, less seminal plasma is favorable [20]. There are basically two ways to do this;the first is to only employ an open-ended artificial vagina to collect the sperm-rich portion of the ejaculate. The second andthe mostpreferred choice is centrifugation. Prior to centrifugation medium, also known as prefreezing extender, which has been preheated to 37.5 °C, the original ejaculate should be diluted with a suitable centrifugation solution. Individual stallions will have a different ideal centrifugation speed and time, but they should be between 300 and 400 x g for 8 and 15 minutes, respectively. A soft sperm pellet is ready to be suspended in a cryopreservation liquid after centrifugation [21]. The gel-free semen must be diluted using a casein- or skim-milk-based extender. This dilution should have a minimum volume-to-volume ratio of 1:1. When working with highly concentrated semen, larger dilutions (2:1) are suggested to reduce damage and sperm loss during the removal of seminal plasma. The diluent medium needs to be heated to 37C before being introduced to the semen in order to prevent cold shock. [22].

The possible harmful effects of centrifugation on the spermatozoa, notably mechanical disruption, have been lessened over time with the adoption of a procedure called cushion centrifugation. In this procedure, a denser mediumsuch as a glucoseethylenediamine tetra-acetic acid(EDTA) buffer, freezing extenders, or iodixanol in wateris placed at the bottom of a centrifuge tube. The diluted semen should then be placed carefully over this cushion. The cushion layer protects the spermatozoa from harm that would come from the spermatozoa being crushed on the hard surface at the bottom of the tube during conventional centrifugation. The spermatozoa travel downhill during centrifugation but then float on top of the cushion layer. Larger centrifugal speeds and forces can be used with cushion centrifugation, which has been found to improve spermatozoa survival rates.(Figure1)

***1.b. semen dilution***

The freezing extender, also known as the cryopreservation medium, is added in step 2 of the process. A variety of commercial casein or skimmed milk-based extenders are available for horse semen. The nutrients necessary for sperm metabolism are provided, they act as buffers to maintain proper pH and osmolality regulation, and they protect the plasma membrane from oxidative and cold shock damage. Additionally, the presence of antibiotics makes these extenders essential[22].Several extender types will be covered later. How much medium is given to the sperm pellet depends on the desired final sperm concentration [23]. Fertilization of the mare's egg requires insemination of a minimum number of healthy spermatozoa. Only semen with a post-thaw motility of at least 35% should be used for insemination since it is estimated that at least 250 million of these sperm cells are progressive motile. However, a disproportionately high percentage of spermatozoa will experience lethal damage during the freezing process if the spermatozoa concentration is too high. This is because channels of unfrozen water with incredibly high salt concentrations persist even at -196 °C. Only spermatozoa that are found in these channels will survive cryopreservation, therefore increasing spermatozoa concentration won't always result in more spermatozoa that made it through the process (Figure 1)[24].

### *1.c. semen cooling*

Cooling and packing of spermatozoa, depending on the extender used and the procedure.Semen can either be packaged into straws right away or first needs to be cooled. The best surface area to volume ratio is made achievable by the use of 0,5 mL straws for the cryopreservation of horse sperm. Because cells in the middle of the straw have a propensity to cool more slowly than cells in the outer layers, this ratio is essential. The more uniform the circumstances are for each cell, the more predictable the outcome of the freezing process is[21]. The lactose-EDTA extender is one that can be frozen without needing to be cooled before. Other extenders need the semen be slowly cooled before packaging. Ideal cooling should take place over a period of two to five hours in order to reach a temperature of five °C(Figure1)[25].

### *1.d. Semen freezing*

Freezing spermatozoa, the sensitive phase of the freezing process occurs when the temperature is changed from 5 to -60 °C. The freezing procedure can be carried out using a device that controls the temperature in the freezing chamber. The straws can also be directly sprayed with liquid nitrogen vapor, but only if the greatest possible surface area is exposed to the rising vapor. To do this, the straws might be placed horizontally. The protocol being used determines the freezing rate; different protocols will be addressed later. As discussed before, freezing rates that are either too fast or too slow can cause lethal damage to spermatozoa (Figure 1)[21].

### *1.e. semen storage :*

Spermatozoa can be stored in liquid nitrogen once they have been chilled to a temperature of -60 °C.The straws should then ideally be kept submerged in liquid nitrogen until they are utilized for insemination(Figure1)[21]

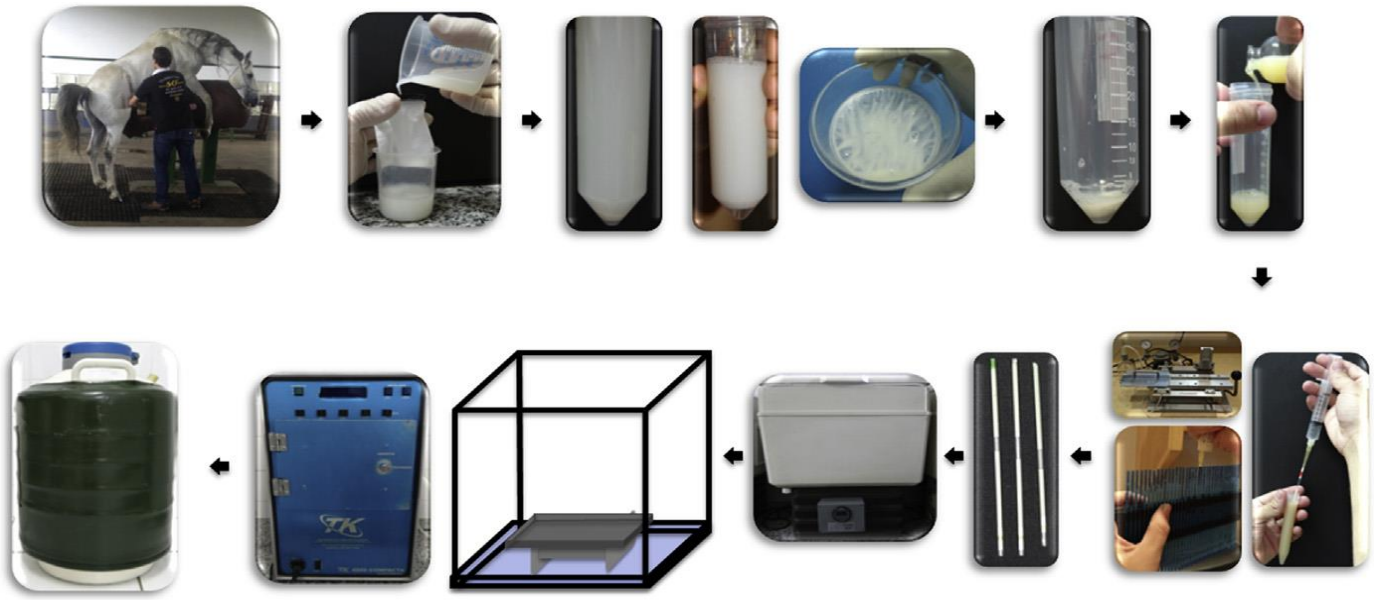

**Figure 1.**The procedures for freezing sperm. The semen is initially collected, filtered to remove gel, and then diluted with media extender prepared from skimmed milk. After seminal plasma has been removed, the sperm pellet is resuspended in freezing extender and frozen [21].

## 2.The benefit of cryopreservation

Cryopreserved semen is preferred over cryopreserved embryos and oocytes for artificial insemination in modern cattle production. This is due to the low cost and minimal effort involved in cryopreserving semen. Despite an increase in the usage of cryopreserved semen, the bulk of equine breeding still employs semen that has been diluted and chilled to 5 °C. Cryopreservation of sperm is usually used to protect important genetic resources [26, 27]. However, the use of this biotechnology in the equine species has been constrained by the regular drop in post-thaw semen fertility of some stallions, as well as the increase in foal expenditures and the demand for increased monitoring of the mare estrous cycle [28, 29]. The spermatozoa are sub lethally damaged by cryopreservation, which can cause them to lose their ability to move, remain viable, fertilize in vivo, maintain the integrity of their acrosomes and plasma membranes, and destroy their DNA [30]. Others claim that sperm DNA integrity is a more accurate measure of sperm activity than sperm metrics like sperm motility rates of fertilization, embryo cleavage, implantation, pregnancy, and live birth [31, 32]. Furthermore, when using cryopreserved semen, it is now possible to keep semen for an arbitrary period of time and ship semen all over the world, expanding the genetic variety accessible [33].

Due to its impact on sperm metabolism and motility, one of the most important factors affecting semen quality is semen pH [34]. Lactic acid is produced by the sperm's glycolytic metabolism, which lowers the pH of the semen sample[35]. Therefore, if semen extender is to improve the quality of frozen semen for horses, more consideration must be paid to it. Different buffers can be introduced to the semen extender to maintain the pH in check while semen is being kept. Variations in buffer capacity may have an influence on the sperm biological system and enzymes (Figure 2)[36].

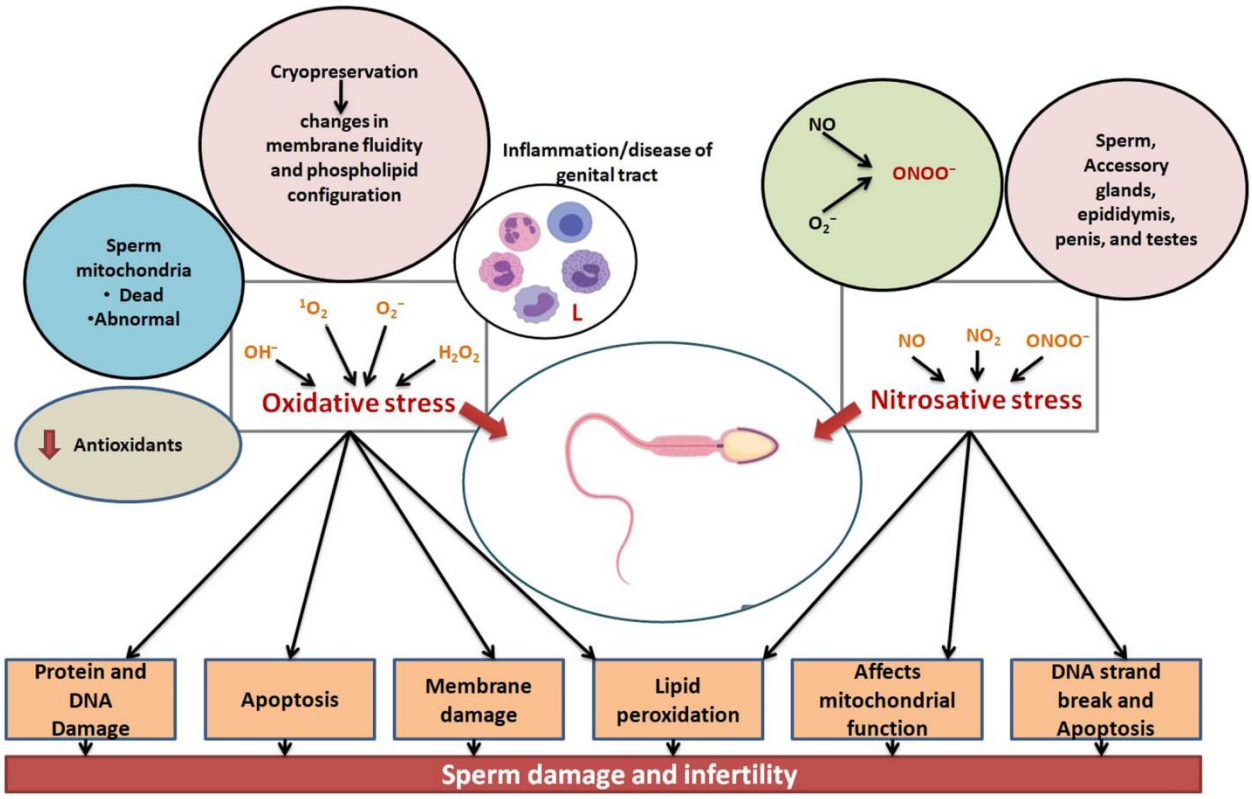


Figure2. Association of Oxidative stress and nitrsosative stress with sperm damage and infertility. Note: $H_2O_2$, Hydrogen peroxide; L, Leucocytes; NO, Nitric oxide; $NO_2$, Nitrogen dioxide; $OH^-$, Hydroxyl ion; $1O_2$, Singlet oxygen; Superoxide anion; $ONOO^-$, Peroxynitrite (Created with BioRender.com).

## 3.Cryoprotectants and its types

The extenders used to freeze sperm typically contain ingredients that stabilize pH, eliminate harmful byproducts of sperm metabolism, provide energy, protect against thermal shock, and maintain the electrolytic and osmotic balance [36]. A wide range ofcryoprotectants(CPOs) and numerous different commercially availablecryoprotectant agents(CPAs ) have been produced in response to market demands. According to current thinking, CPOs reduce osmotic stress exposure, stabilize biomolecules and their structures, and reduce the impact of reactive oxidative species (ROS). The perfect CPA would not cause cryoinjury or osmotically dry the cell, and it wouldn't be poisonous. Reduced intracellular freezing, reduced cellular damage from the freezing environment, and increased cell survival should all be the objectives of a CPO. Cryoprotectants must also be included in the extenders to stop intracellular and extracellular ice from forming various industrial extenders. There are various options available with lactoseEDTA (Animal Reproduction Systems, Chino, CA),Institute National de la Recherche Agronomique (INRA) freeze (IMV), and BotuCrio (Botupharma, USA) being the most popular. Semen from stallions has been frozen using a variety of cryoprotectants. These cryoprotectants can be classed as intracellular or

extracellular, penetrating or nonpenetrating [37]. The intracellular cryoprotectants work by colluding or by their capacity to bind water. The extracellular substances protect the sperm cells by taking advantage of osmotic effects to create a hypertonic environment that induces water to migrate out of the cells, drying the sperm and reducing the possibility that ice crystals will form inside the cells. So, the harm that ice formation could have caused to sperm is prevented. Without the need for nonpenetrating cryoprotectants to penetrate, the sperm cells are well protected during freezing. Some examples are milk and egg yolk;egg yolk has been regularly used for the past 65 years as extenders for the cryopreservation of mammalian semen and to protect sperm from thermal shock due to the presence of low-density lipoproteins. These lipoproteins adhere to the cell membrane upon freezing and take the place of the missing phospholipids. To protect the sperm cells and prevent the cell membrane from rupturing, the egg yolk appears to temporarily alter the phospholipid composition [38]. Egg yolk is typically utilized at a 20% concentration. Another nonpenetrating cryoprotection method that sugars offer is an osmotic pressure dehydration of the cells, which lowers the amount of intracellular water that may potentially form ice. Additionally, sugars interact directly with cell membranes to preserve the plasma membrane during freezing and thawing as well as provide energy for the sperm during incubation [39].

Glycerol is the most prevalent cryoprotectant used for the cryopreservation of semen. Glycerol reaches the cell membrane through passive diffusion and remains there with the cytoplasm. Although water passes through the membrane more quickly and dehydrates the cell, these chemicals still pass through it until equilibrium is achieved. In addition to its negative osmotic effects, glycerol may also have direct practical methods and an intellectual framework [40]. An important advancement in cryopreservation was bull spermatozoa's usage of glycerol as a cryoprotective agent [41]. In the cytoplasm of sperm cells, glycerol takes the place of water, reducing the growth of intracellular ice crystals. Glycerol has not, however, been as effective in the cryopreservation of stallion spermatozoa, and there is a wide range of success amongst different stallions [42]. Stallion spermatozoa are more negatively impacted by ethylene glycol poisoning than bull spermatozoa are. Other cryoprotectants, however, have only shown to be as effective as glycerol or less effective [27]. Smaller glycerol concentrations are more effective in preserving sperm cells than 7% glycerol concentration, which is required for bull spermatozoa. Egg-yolk extenders have been tried with glycerol at various doses andconcentrations [43, 44]. According to recent studies, stallion spermatozoa are substantially more permeable to lower molecular cryoprotectants (CPAs) like dimethylformamide (DMFA) than to glycerol [45], which results in reduced osmotic stress than the latter. Using extenders based on this CPA may lessen cryodamage if osmotic stress is, in fact, the primary cause of damage for stallion spermatozoa [46]. In an effort to enhance current cryopreservation procedures and better understand the physiology of spermatozoa subjected to freezing and thawing, numerous laboratories have tested various extenders or cryoprotectants throughout the years [47]. Cryopreservation of spermatozoa can

preserve human and domestic animal genetic material[48]. It is well known that freezing and thawing sperm can harm them in different ways. The sperm's motility, viability, and capacity to fertilize are all reduced as a result of these harms, which also include disturbances to the sperm organelles, alterations to membrane fluidity, and changes in enzyme function [49]. Cell mortality and subcellular damage that occur during freezing and thawing have been attributed to two basic mechanisms, namely the creation of intracellular ice crystals and osmotic stress [50].

The low freezability of equine sperm has been linked to a number of issues, including the freezing method [51], the make-up of the freezing extender, and the type of cryoprotectant used [27]. One of the key elements affecting the efficacy of cryopreservation is the composition of the extender used to dilute the semen before freezing [52]. The most effective diluents used with stallion semen for artificial insemination comprise skim milk, despite the fact that a number of extenders and their modifications have been produced [53]. Milk is a biological fluid with a complicated chemical make-up that includes elements that are either good or bad for spermatozoa. Milk fractionation has made it possible to create fractions of pure protein. Phosphocaseinate and lactoglobuline were discovered to be the two that helped equine spermatozoa survive the longest when they were stored at low temperatures [54]. In general, extenders containing specified milk protein (EquiPro) andINRA96 sustained equine spermatozoa's progressive motility throughout storage better than extenders made of skim milk [55]. After thawing, microscopic quality based on motility, functional, and morphologic characteristics is crucial. However, freezing and thawing procedures can damage the sperm cell membranes, including the plasma, acrosome, and mitochondria. Aftercryopreservation, diminished motility is primarily caused by cold shock. Typically, it causes edema, acrosomal membrane protuberance, and other symptoms [56, 57].

Organic DMSO, which contains sulfur, readily passes cellular membranes. Because of DMSO's rapid penetration, water is moved from the intracellular fluid to the extracellular environment more quickly. Due to the unpredictability in stallion sperm, a little quantity of this powerful chemical is commonly used in conjunction with glycerol or another CPA as an added speed component and to help stabilize the cell before freezing [58]. However, DMSO is favored and used for cattle at far larger quantities than necessary with some species. It's likely that animals with semen that responds better to DMSOfreezing do so because glycerol acts as a contraceptive for them. Using DMSO as a significant CPA has been advantageous for mice, rabbits, and a variety of fish, including zebra fish, carp broodstock, seven-band grouper, and mutton snapper [59]. With respect to glycerol forCP, rabbit sperm tend to function best when DMSOis present in significant amounts. According to some studies, this could be caused by a deficiency in the glycerol transporter and water channel protein aquaporin 7 (AQP7). Some non-human primate’s family members have also used DMSO for semen CP. The Cynomolgus monkey (Macaca fascicularis) and the Rhesus monkey (Macaca mulatta), both members of the macaque family, have produced contradictory results. While the Rhesus failed after utilizing a stair step increasing trial of DMSO to glycerol, the Cynomolgus semen was successfully

frozen when DMSO was in an equivalent proportion to glycerol [60].

Amides have demonstrated to be a generally advantageous CPA, as they have been shown to produce fewer negative effects than when glycerol is utilized only as the CPO [61]. Poor freezers have needed a lot of attention since stallions were divided into various freezing classes. Amides have improved this class's freezing potential while also reducing the overall negative effects of glycerol [62]. While glycerol continues to be the primary CPA utilized for stallion sperm, the inclusion of amides may lessen sperm cell injury in part because of their decreased viscosity and molecular weight [63].

Dimethylformamide (DMF) has been shown to enhance post-thaw motility and keep cellular membranes intact, both of which may greatly enhance semen freezing potential [64]. Because they have smaller molecular weights than glycerol and can penetrate stallion sperm more efficiently as a result,DMF andmethylformamide(MF)are less dangerous than glycerol because they experience less swelling when they equilibrate in diluents that contain amide [65]. However, it appears that the only amides that might have cryogenic effects are DMF and MF. Other amides have negative effects on semen's ability to act as a cryoprotective. Since stallion sperm is very unique, as was already said, research on other CPAs that could help to preserve frozen sperm has exploded [66].The discovery of MF and DMF as agents has considerably helped the business and encouraged investigation into further potential replacements. Experts in horse cryobiology have been working for the past 30 years to lessen the harm brought on by freezing and thawing, enhance membrane integrity, and boost progressive motility. [67].Throughout that period, the majority of researchers investigating alternative CPAs have concentrated on reducing damage brought on by freezing and thawing. Recent studies have demonstrated that reversible binding of exogenous phospholipids and the presence of liposomes, which fuse to the plasma membranes of sperm, shield sperm against damage. Methyl-cyclodetrin and cholesterol have been shown to lower membrane transition temperatures, which reduces cryoinjury, upholds cellular membrane integrity, and improves post-thaw motility [68].

Lipids: Previous research has shown that CP can result in the loss of from the membrane, which then causes peroxidation and creates reactive oxidative species [69]. Since they have been connected to both the oxidation and the protection of lipid bilayer infusions, lipid bases have a variety of functions. As a result of the generation of ROS and the recruitment of lipids from the membrane, which results in lipid rearrangement within the membrane and further oxidation, the addition of a lipid-based CPO may make the sperm membrane unstable. Acrosomal activity and motility may be impacted by increased peroxidation as well.Due to osmotic stress and relative temperatures, sperm are vulnerable to cold shock damage, which may in turn cause underlying damage to the membrane's integrity. It is unknown exactly how lipids preserve spermatozoa during freeze-thaw, and more research is required to clarify this [70]. Therefore, there are a few more concerns that must be addressed If lipids are to be included to create a more saturated CPO for semen preservation during cryoprotection. Numerous studies have demonstrated the important function ROS play in male infertility. Furthermore, lipid phase shifts

that result in leaky sperm membranes and compromise membrane integrity have been directly linked to cold shock damage [71]. However, ROS has shown to be a two-edged sword. Although their negative effects are well known, sperm normally undergo a number of physiological processes at low concentrations, such as capacitation, the acrosome response, and binding to the Zona pellucida [70].

Methylformamide has also been employed, and in our experience, because of its lower molecular weight and viscosity, it doesn't harm sperm as much as glycerol does. In stallions with semen that has a fair freezability (referred to as "good freezers"), the use of extenders including dimethylformamide and methylformamide may not significantly enhance post-thaw sperm motility, but it does increase the fertility of the frozen semen. When compared to extenders containing glycerol, the use of dimethylformamide and methylformamide improves sperm motility and fertility in stallions with low semen resistance to cryopreservation (often known as "bad freezers") [71]. Sperm are better protected when multiple cryoprotectants are used in combination than when only one or two are. A commercial extender (BotuCrio USA), which combines methylformamide and glycerol, has been employed preferred by a number of equine reproduction facilities in Europe, the US, and Brazil. Immediately following the removal of the seminal plasma, the semen must be combined with the extender used in cryopreservation [45]. The total quantity of sperm in the recovered semen sample must be quantified in order to identify the precise volume of extender required. Stallion sperm is typically cryopreserved at a concentration of 200 to 400 million sperm/mL, or 100 to 200 million sperm per 0.5 mL straw. After the semen has been packaged, the samples are cooled to 5 °C. The length of time needed to reach equilibrium at 5 °C varies depending on the type of extender being used and the manufacturer's recommendations. In order to avoid the production of intracellular ice crystals, The freezing procedure must be quick enough to protect sperm from supersaturated conditions while still being slow enough to allow cell dryness [65]. First, the semen straws must be cooled from room temperature to 5 °C at a rate of 3 °C to 5 °C per minute in order to start the freezing curve., and then they must be frozen to 196C at a rate of 20 °C to 50 °C per minute. The process to freeze stallion semen has specifics for different extenders. The lab advises cooling INRA 96 for two hours at 5 °C before beginning the freezing process. A shorter equilibration period (20 minutes at 5 °C) is advised for BotuCrio. Following the equilibration, the suggested freezing curve is comparable between extenders [44]. The ability of a compound to act as an effective cryoprotectant depends both on its ability to protect cells from freezing damage and on its nontoxicity to cells. The cryoprotective capacity of a compound depends both on the number of one electron pairs the compound contains, the spherical symmetry of the one electron pair, and the solubility of the compound in water [45].

In a previous study[72], thee used honeybee in Arab stallion semen extender as an energy substrate, for its advantageous antioxidant impact on sperm membrane integrity, and none penetrate cryoprotectants. 1%, 2%, 3%, 4%, and 5% of honey have been utilized as cryoprotectant extenders for Arab stallions. According to the previous study's findings, once Arab stallion sperm were cryopreserved, the integration of

various doses of the honeybee freezing extender boosted sperm motility, viability index, and membrane and acrosome integrities.When honey was included in the extender (2%, 3%, and 4%) at 0, 1, 2, and 3 h after freezing, sperm motility measurements were significantly higher than the control (0%). In comparison to control, most honey concentrations (2%, 3%, and 4%) had considerably greater viability indices, percentages of sperm with intact membranes, and intact acrosomes. In contrast to the means recorded for the control andhemispherical(HEMI) (1%, 2%, and 5%), the enrichment of the mINRA-82 with the honeybee (HEMI 3%) revealed the greatest significant mean records for the sperm motility, viability index, theHypoosmotic swelling HOS positive cells, and the acrosome integrity. Also, researchers[73,74] reported that spermatozoa motility and sperm quality in goat, ram, and cattle bull semen, respectively, when honey was included in cooling and freezing extenders.

**Conclusion**:

Cryopreservation is very important for elongation the life span of sperm and improve the genetic characters of animals. Cryoprotectants were used for decreasing the cryo-injury of sperm, so should be choose the best cryoprotectant for semen sample.

**Conflict of interest**

There is no conflict of interest, according to the authors.

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**الملخص العربي**

**حفظ السائل المنوى لفحول الخيل بالتبريد: دراسات سابقة**

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يعد حفظ الحيوانات المنوية لفحول الخيل بالتبريد عملية معقدة تتطلب التحكم في العديد من العوامل للحصول على النتائج المرجوة. إن المواد المخففة المناسبة، ومعدلات تخفيف الحيوانات المنوية، ومعدلات التبريد، ومعدلات الذوبان مطلوبة لضمان الحد الأدنى من النجاح. لتحقيق أقصى قدر من استعادة الحيوانات المنوية بعد الذوبان، وبالتالي الخصوبة، من الضروري أيضًا معرفة شاملة بفسيولوجيا الحيوانات المنوية المعقدة لكل نوع. يتم استخدام السائل المنوي المحفوظ بالتبريد بشكل أقل بكثير في التلقيح الاصطناعي للخيول (AI) مقارنة بالسائل المنوي الطازج أو المخزن المبرد. يرتبط حفظ السائل المنوي بالتبريد أيضًا بارتفاع الأسعار. لقد استفادت تربية الحيوانات بشكل كبير من استخدام السائل المنوي المحفوظ في التلقيح الاصطناعي خلال العقود السبعة الماضية. وكانت الحيوانات المنوية لدى الثدييات من بين الخلايا الأولى التي تم حفظها بالتبريد بشكل فعال.

وبالتالي فإن الفعالية الاقتصادية للتلقيح الاصطناعي التجاري باستخدام السائل المنوي المحفوظ بالتبريد تتأثر بعدد جرعات التلقيح التي يتم الحصول عليها لكل قذفة، وأيضًا واحدة من أكثر المواد تحديًا في صناعة السائل المنوي للخيول هي الحفظ بالتبريد بشكل فعال دون موت الحيوان المنوي أو تلف الغشاء. تختلف الفحول في قدرة الحيوانات المنوية على تحمل البرد. ومع ذلك، لا يزال من غير الواضح مدى تأثر هذا الاختلاف بالسلالة. تشمل طرق الحفظ بالتبريد الشائعة للحيوانات المنوية الغمر المباشر في النيتروجين السائل والتجمد بدون تكون كرات ثلجية . ومع ذلك، هناك احتمال أن يحدث التلوث الميكروبي عند التجميد المباشر للحيوانات المنوية في النيتروجين السائل. لذا فإن الحفظ بالتبريد يهدف إلى حماية الحيوانات المنوية وإطالة صلاحيتها. المواد الواقية من البرد هي مواد ذات وزن جزيئي منخفض ونفاذية للغاية. تتم حماية الحيوانات المنوية من أضرار التجميد عن طريق تبلور الجليد وذاك من خلال تقليل درجة حرارة تجميد العينات. تشتمل أشهر المواد الواقية من البرد على الجلسرول، وجلايكول الإيثيلين، وثنائي ميثيل سلفوكسيد، و1،2-بروباندیولوالديمسو. ولذلك الهدف من هذا المقال هو الشرح بالتفصيل خطوات الحفظ بالتبريد وأهميته وأنواع المواد الواقية من التجميد التي تضاف إلى السائل المنوي لفحول الخيل.