The Advancements and Obstacles of Stallion Semen Cryopreservation

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Abstract

Cryopreservation is a process that preserves intact living cells, tissues, organelles with the use of very low temperatures, extending their lifespan. The ability to cryopreserve sperm cells has facilitated the accessibility to semen and for decades has played a major role in dairy cattle production, thanks to its ability to increase the rate of genetic selection. Additionally, frozen semen has been a catalyst for the development of reproductive techniques. Although this technology has been a milestone in reproduction, it has several limitations that to this day, influence pregnancy rates. These limitations are associated with a combination of molecular and biochemical changes that occur due to the different stages of cryopreservation. These alterations are responsible for compromising the fragile physiology and anatomy of the sperm cell. Putting spermatozoa under stress like freezing, reduces their ability to survive in the female reproductive tract, thus affecting the chances of fertilizing an egg. Despite all this, several experiments and studies over the years have taken place to determine the best protocols and technical adjustments to improve the fertilizing efficiency of frozen-thawed semen, while still benefiting from the conveniences that come with frozen preserved semen. (Medeiros et al., 2002)

Introduction

History & Significance of Cryopreservation

Lazaro Spallanzani was one of the first to perform successful artificial insemination (AI) in 1780 on a bitch with fresh semen. John Hunter followed this with the first human pregnancy achieved by AI.

While the development of AI occurred in the late 1950s and early 1960s, the first offspring was born from frozen semen. After this, the demand for long term storage of bull semen rose, driving scientific investigation of cryopreservation further, and from there, sperm cryobanks began popularizing.

The advancing technology of cryopreservation provided considerably better results over time; this was mainly achieved by refining critical points in the protocols, such as temperatures, cooling and freezing periods; however the development of semen extenders, and not to mention the pivotal introduction of glycerol as a cryoprotectants by Polge et al in 1949, made a significant which was used to freeze fowl and bull sperm. (Skaife , 2013)

Cryopreservation has also played a beneficial role in human medicine, allowing for reproductive management of those patients undergoing chemotherapy or surgeries that come with ejaculatory

disorder side effects. From a veterinary point of view, the technical methods of cryopreservation were further investigated and refined with the aim of applying cryopreservation to several other species. Cryopreservation expanded the use of AI and IVF in various domestic animals, which in turn increased the rate of genetic selection and production rates, especially noted in dairy cattle. (Medeiros et al., 2002) On the other hand, it provides a possibility for preserving genetic material of endangered species and breeds, or in the case of unexpected death or emergency castration. (Ďuračka et al., 2023) The use of cryopreserved semen in equine reproduction has increased significantly over the years thanks to AI restrictions being lifted by breed registries. Stallions in particular are selected mainly for phenotype, conformation and athletic performance (Alvarenga et al., 2016)

This paper will focus on the advancements and developments made over time to improve the outcomes of cryopreservation in equine reproduction.

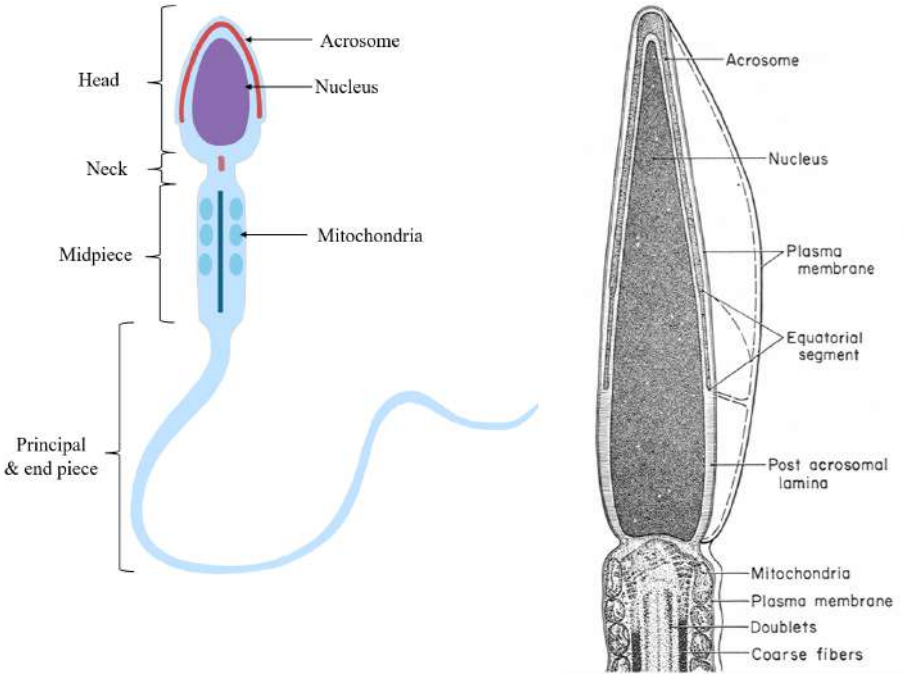
1. Physiology of Equine Spermatozoa

For cryopreserved spermatozoa to successfully fertilize an egg, at least four physiological components and functions must remain after the semen sample has been thawed:

1. Metabolism
2. Progressive motility
3. Enzymes in the acrosome
4. Proteins on the plasma membrane

The hydrolytic enzymes found in the acrosome are released as a consequence of the acrosome reaction; their function is to penetrate through the cumulus cell layer and zona pellucida (ZP) of the oocyte (Berruti and Paiardi, 2011). Proteins found on the plasma membrane play a protective role for survival in the environment of the female reproductive tract. Another vital function of plasma proteins is the attachment of the sperm to the egg plasma membrane during fertilization. (Amann and Pickett, 1987)

Spermatozoa are highly sensitive cells with limited survival, due to its own inherited features that depend on and are affected by, the extracellular environment of the male and female reproductive tract. The anatomy of spermatozoa consists of very few vital organelles and an almost absent cytoplasm; hence why it relies on its surrounding environment to absorb the necessary molecules for maturation and maintenance of cell membrane functions.



- **Nucleus**: genetic information in the form of highly condensed chromosomes, in order to stop transcription and prevent replacement of proteins/
- **Acrosome**: contains enzymes that aids the sperm penetration through the oocyte during fertilization
- **Mitochondria**: provides ATP to maintain motility.
- **Endoplasmic reticulum:** very little, to maintain membrane integrity

Fig. 1 left: Stallion Spermatozoa, illustrated by Cristina Martinez, 2024
Fig. 1.2 right: cross section of Stallion spermatozoa, illustrated by Amann and Pickett, 1987

The sperm cell undergoes a series of maturation and structural changes as it advances from the male to the female reproductive tract environment. These biochemical mechanisms are defined as capacitation and acrosome reaction.

The acrosome reaction is a biochemical and structural process concerning the:

1. Fusion of the plasma membrane and external acrosomal membrane
2. Vesiculation of the membrane and expulsion of acrosomal enzymes
3. Internal acrosomal membrane becoming exposed

In contrast, capacitation entails a cascade of biochemical and molecular transformations that aid the penetration to the oocyte. As soon as the sperm exits the epididymis via ejaculation, a series of modifications occur in the plasma membrane, which stimulates capacitation. This process is not complete until ovulation; however, once it has been activated, the lifetime of the sperm becomes very limited. This means that spermatozoa with a high fertilizing capacity complete capacitation when there is an adjacent unfertilized oocyte present. This is of special importance because it may provide hints

regarding the origin of some of the damaging insults caused by cryopreservation. Sperm cells are cryopreserved during the first phases of capacitation; once they are thawed, they reach an advanced stage of capacitation. (Medeiros et al., 2002)

Capacitation is dependent on the sperm cells intracellular conditions and its interactions within the female reproductive tract. It ecompasses a series of changes to the sperm membrane and intracellular ionic conditions. Firstly, decapacitation factors on the surface of the sperm are released; these are important because they are in control of capacitation and are responsible for the mobility of membrane components. This is followed by the adsorption of proteins originating from the female tract onto the sperm. The conformation of the membrane changes; these consist of a transformation of the molecular and integral proteins, and the cholesterol to phospholipid ratio. There is a loss of cholesterol in the plasma membrane that triggers a change in the membrane stability, causing it to become more fluid and permeable. In this state, the membrane is capable of fusing with the external acrosomal membrane.

There is a direct correlation between the cholesterol to phospholipid ratio and the time required for capacitation to occur. Studies carried out on bovine sperm depleted of cholesterol, resulted in faster acrosome reaction and fertilization rates. On the other hand, when semen was incubated with cholesterol, the fertilization rates were significantly delayed. Scientists discovered that cyclodextrins (CD), when applied to the incubation medium, remove cholesterol from the membrane, which induces capacitation at a faster rate.

Capacitation not only affects the sperm cell structurally, but also its intracellular physiology. It is responsible for the deregulation of ions (increased internal Ca+, Na+ and pH) and production of reactive oxygen species (ROS) (superoxide anion, hydrogen peroxide, nitric oxide). Spermatozoa physiologically produce ROS which play a role in the capacitation and acrosome reaction: lipid peroxidation aids sperm binding to the ZP. Nonetheless, ROS are highly reactive molecules and are therefore toxic by nature. ROS inactivate proteins, damage DNA and cause peroxidation of unsaturated lipids; the latter results in destabilization of the plasma membrane, disrupting the enzymes bound to the membrane, inevitably leading to apoptosis. The way in which sperm produces ROS is not fully understood, however their effect can be lessened by detoxifying agents.

It is hypothesized that the modifications of the cholesterol content in the membrane, induced by cryopreservation, lead to premature capacitation.

2. Basic Principle of Cryopreservation

Spermatozoa of different species are unique in shape, cell volume and size of organelles - hence, species-specific cryopreservation protocols have been developed, where certain standards have been set to accommodate these unique species differences. The same 50% motility attained with fresh semen, has been achieved with cryopreserved semen for the majority of species. Despite this, the proportion of motile sperm in the insemination dose still remains variable between species. (Medeiros et al., 2002)

Below is a summarized overview of the stages involved in cryopreservation. The order and specifications of each step varies between species, individual stallions, or geographical regions.

1. **Semen Collection:** before collection, the penis is washed with warm water, paying close attention to the urethral fossa. Bactericidal and spermicidal disinfecting solutions must be avoided. Traditionally, stallion semen is collected either using a phantom or using a teasing mare for sexual stimulation. Ejaculation in stallions is triggered with the use of an artificial vagina (AV). (Pesch & Hoffmann, 2007) Several models exist: Missouri, Colorado, Hannover (EU), Botucatu, and more. See figures 2.1 - 2.3 for examples of AV models used in Al Hawajer Stud farm, Sharja.

   The artificial vagina is filled with hot-warm water to aid the stimulation for ejaculation. For hygienic purposes, the AV is lined with a disposable plastic cover to prevent transmission risk of pathogens, such as Pseudomonas. It is important to mention that latex liners can be toxic to spermatozoa and care should be taken. (Alvarenga et al., 2016) Finally, the AV may be lubricated using a specific neutral lubricant; however, many AI centers avoid lubricants because of the potential risk of inducing sperm damage.

Fig. 2.1 Colorado

Fig. 2.2 Missouri

Fig. 2.3 Modified Colorado

Fig. 2.1 - 2.3: artificial vaginas utilized in Al Hawajer, UAE, photographed by Cristina Martinez, 2023

2. The semen sample is **filtered** to remove the gelatinous portion and any ejaculate debris.
3. Once the sample is gel free, it is **diluted with a primary extender** (e.g. skim milk or casein based extender), at 1:1 or 2:1 ratio, depending on how concentrated the sample is. Afterwards, the sample can be evaluated for motility, viability, and sperm concentration.
4. **Centrifugation** concentrates sperm cells from the seminal plasma (fluid produced by accessory glands). The rate of centrifugation is dependent on the species or individual stallion. However the force and duration of centrifugation can damage spermatozoa motility and integrity, hence care must be taken when setting the rate. Too high forces cause adhesion while very low forces reduce sperm recovery. Dr Marco Alvarenga et al. carried out an extensive study on cryopreservation advancements in stallions in 2016, where he found that 600x g for 10 minutes was a suitable and safe centrifugation rate. After centrifugation the sperm sample should be soft and easily resuspended; if the sperm pellet is compact, it signals that spermatozoa were crushed due to too high centrifugal speed or time. (Graham, 1996)
5. The supernatant is removed with a syringe or vacuum pump and is resuspended in the desired **freezing extender.**
6. **Packaging of semen in plastic straws** of 0.5 ml or 0.25 ml. Makrotübs (4 ml) and Midipaillettes (0.5 ml) are commonly used for stallion semen, however Makrotübs are no longer accepted due to semen leakage upon thawing. (Pesch & Hoffmann, 2007) An air bubble must be present in the middle of the straw to permit fluid to expand during freezing and therefore prevent the straw from bursting.
7. **Cooling** must be carried out at an equilibrium, following a freezing curve. The rate should be slow enough for cells to sufficiently dehydrate and prevent intracellular freezing, but rapid enough to avoid dehydration caused by an exposure to hyperosmotic conditions. The optimal cooling rates vary between species, and may be particular for each extender. (Medeiros et al., 2002) For instance, the cooling protocol for INRA 96 is 5°C for 2 hours prior to freezing, whereas for BotuCrio 5°C for 20 minutes is recommended. (Alvarenga et al., 2016)
8. **Freezing** equine semen may be done with styrofoam coolers or programmed freezers. Both methods compared in studies found no significant difference between either. For example, the straws may be placed on a rack above a styrofoam container filled with liquid nitrogen. Generally temperatures reduce from 4°C to -140°C, but this is dependent on the protocol.

9. **Thawing** can be done in several ways with different temperature rates ranging from 46°C for 20 seconds to 37°C for 1 minute. Once thawed, the semen can be examined for motility (total, progressive), vigor of movement, morphology and number of spermatozoa per straw. It's important not to repeat dilution of the semen after thawing to prevent osmotic damage; this is an exception for the following step.
10. A final step of the thawing process involves removal of glycerol if it was incorporated. This is done by diluting the sperm in a medium free of glycerol, or simply by insemination itself since the environment of the female reproductive tract will dilute the glycerol.

Evaluating Semen Quality

Prior to freezing, the traditional methods used to analyze semen quality are limited to visual assessment using microscopy. This method is subjective, and thus yields results with high variability. Fortunately, with computer-aided sperm analysis (CASA), semen samples can be evaluated more objectively and accurately for the following parameters: motility (%), progressive motility (%), velocity, linearity (%), and lateral head displacement (μ).

Although spermatozoa motility and progressive motility are more common parameters used to predict fertility, studies carried out in humans proved that the velocity was strongly correlated to fertility rates. Velocity is the only parameter that correlates with that population of sperm that possess a functional mitochondria and an intact plasma membrane; this was deducted with the use of flow cytometry. (Burns and Reasner, 1995) According to research by Burns and Reasner, 1995, they suggest that using motility as a measurement for sperm quality and linking this to fertility rates is emphasized more than appropriate.

Criteria of spermatozoal survival

a. Percentage of motile spermatozoa carried out by visual examination
b. Structural and functional integrity (semi-quantitative analysis of acrosomal damage by microscope)
c. Pregnancy rates with cryopreserved semen

Temperature Control during Cryopreservation

In order to cease metabolic and chemical reactions from taking place, spermatozoa have to be cooled and then frozen to temperatures below -13O°C. Between 4°C and 5°C is the acceptable cooling temperature. (Bustani & Baiee, 2021) Even though metabolic processes are reduced below 4°C, the remaining metabolic activity in the sperm cell will lead to death, since they rely on catabolic processes to function. (Medeiros et al., 2002) The side effects of cold shock can be prevented by adjusting cooling rate, as rapid temperature drops inflict damage to the spermatozoa. As discussed in earlier sections, rapid cooling rates trigger unfrozen intracellular water to diffuse out towards the high extracellular solute concentrations, causing cellular dehydration. Slower freezing rates cause the spermatozoa to become dehydrated enough that intracellular ice crystals cannot form, however the dehydrated state predisposes the cell to a harmful increase of intracellular solutes. (Graham, J.K., 1996.)

The ideal freezing rate should be fast enough for the extracellular water to freeze, without causing intracellular ice to form. At -5°C the intracellular and extracellular water is in an unfrozen supercooled state. Between -5°C and -lO°C, ice forms extracellularly and intracellular water remains supercooled. At temperatures below -lO°C, intracellular water freezes, forming ice crystals which puts the spermatozoa into a critical state, because it imposes mechanical stress, disrupting the cellular structure. When intra- and extracellular environments are maintained at an equilibrium, it induces cellular dehydration, which leads to cell to shrinkage and its membrane to collapse irreversibly. (Medeiros et al., 2002)

Once cooled below the critical zone (-15°C and -60°C), the spermatozoa become inert and the sample can be plunged into liquid nitrogen for storage. In this state, cellular metabolism stops, and any risk of damage caused by high salt concentrations is reduced.

The thawing temperature rate is another influential factor that must be considered; this is dependent on the freezing rate. If the cooling rate is rapid, the thawing rate must also be fast. Likewise, if a slower cooling rate is applied, the thawing rate must also be slow. The theory behind this is as follows: rapid warming rates melts the ice quickly, diluting extracellular solutes which pulls water into the spermatozoa instantly, thus damaging the cell. Slower thawing permits the water to diffuse back at a

slower rate, so that intracellular concentrations can return to equilibrium. (Graham, J.K., 1996.) When fast cooling rates but slow thawing is applied, intracellular ice re-crystallizes into larger, more harmful crystals.

3. Cellular Changes Induced by Cryopreservation

In order to understand the impact of cryopreservation on semen quality, it is necessary to overview the biochemical, molecular and physiological changes that occur to the sperm cell during cryopreservation.

Throughout the several studies carried to examine spermatozoa post-thaw viability and quality, two main cellular changes can be highlighted: structural and molecular (Yánez-Ortiz et al., 2022). It is important to comprehend the cellular injury that occurs to the sperm, because it provides information on which freezing steps must be investigated further, in hopes to implement measures that will bring forth higher semen quality post-thawing.

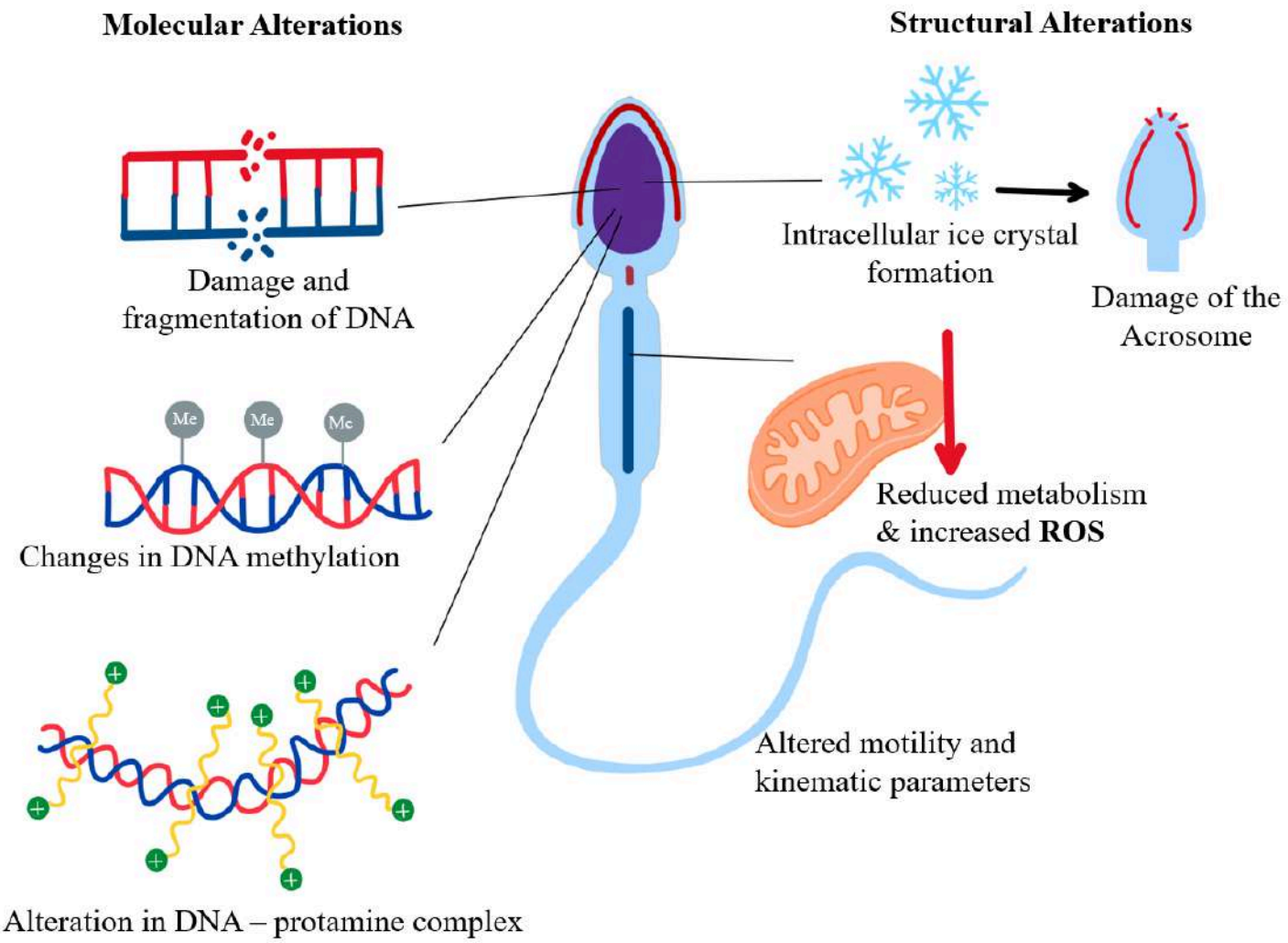


Fig. 3 Molecular and Structural alterations that occur to the spermatozoa during cryopreservation (Illustration by Cristina Martinez 2024, reference from Yánez-Ortiz et al., 2022)

Structural Alterations

There are a series of thermal transitions throughout cryopreservation (body temperature, cooling, freezing, and thawing) that sperm cells are not adapted to endure; these cells are inherently sensitive to temperature change. (Medeiros et al., 2002)

During cooling, temperatures drop below 18°C. This temperature change stimulates a physical shift in the spermatozoal membrane, which involves a rearrangement of lipids and proteins, described as a lateral migration. The membrane phospholipids rearrange from a fluid crystalline state into a gel state. This disrupts the lipid bilayer as it forms microdomains of non-bilayer forming lipids, thereby affecting protein surrounding environments. During thawing, this conformational change causes opposing membranes to merge, having two consequences: interrupting the function of cellular proteins and modifying the membrane permeability. (Medeiros et al., 2002) It is possible to prove the disrupted membrane permeability by staining the semen sample with dyes that originally do not permeate the intact membrane; therefore if the plasma membrane is damaged, intracellular staining will be obvious. (Medeiros et al., 2002)

These physical changes also involve a loss of cholesterol and phospholipids in the membrane, predisposing spermatozoa to cellular **cold shock**. The lipid distribution in the membrane determines the cell's ability to survive injuries inflicted by cooling temperatures.

Furthermore, research has demonstrated that sperm membrane lipid composition is correlated with the ability of the spermatozoa to tolerate the processes during freezing. Interestingly, it has been shown that there is an increase in sperm membrane polyunsaturated fatty acid (PUFA) content during the breeding season. In 2020, scientists were able to demonstrate that there is an active lipid metabolism in the stallion testis and epididymis, which likely has an effect on the lipid profile of equine sperm plasma membrane. (Aurich et al., 2020)

Physiological Alterations

The spermatozoa is predisposed to various physiological changes during the cooling phase, including cellular dehydration and formation of hazardous intracellular ice crystals. Sperm cells **shrink** when exposed to the **hyperosmotic** conditions of frozen extracellular water. When cooled below 0°C, ice

crystals form in the extracellular space. These ice crystals have scattered, unfrozen channels containing high concentrations of solutes, creating a hyperosmotic environment for the sperm cell.
On the other hand, cryoprotectants also have an **osmotic** effect on spermatozoa, particularly during the addition or removal of them. Cryoprotectants have low membrane diffusion rates compared to water, therefore they create an osmotic gradient across the cell membrane. This gradient increases the sperm cell volume, which is dependent on the amount and type of cryoprotectant used: high amounts of cryoprotectants result in larger cell volume. Additionally, certain cryoprotectants with low diffusion rates may also increase cell volume.

In the beginning of the cooling phase, intracellular water does not freeze immediately, which means it can circulate out to the extracellular space, leading to cellular dehydration. As a consequence, the cell **shrinks**. If intracellular water cannot diffuse out fast enough, intracellular ice crystals will form, which are damaging to the sperm. With fast cooling rates (> -60°C /min), there is not enough time for intracellular water to diffuse out of the sperm cell before it freezes into harmful ice crystals. (Loomis and Graham, 2008) Delayed cooling rates allows more time for intracellular water to escape and cause cellular dehydration; in this state, ice crystals cannot physically form inside the cell. Although the cell is not at risk of injury from ice crystals in this scenario, the intracellular concentration of solutes increases, which may be harmful. (Graham, 1996)
Moderate cooling rates such as -25°C to -40°C, the cell is still predisposed to dehydration as intracellular water diffuses out.
The spermatozoa **metabolism** decreases with each 10°C drop. Metabolism is completely halted when cooled below the critical zone: -15°C to -60°C. In this state, semen lifespan can be extended and stored indefinitely. (Graham, 1996)

Molecular changes

In various species, molecular changes occurring as a result of cryopreservation seem to be more severe than physical damages, as it involves DNA damage. The thermal stress of freezing and thawing, oxidative stress and mechanical stress caused by cellular contraction, all disrupt the disulfide bridges between cysteine and compacts the chromatin, which in the end affects the integrity of the DNA double helix. (Yánez-Ortiz et al., 2022) Any damage to the chromatin ultimately fragments the DNA. This is why the molecular damages are believed to be reflected upon the conception rates. Not to

mention, the temperature decrease induces excess production of **ROS** consequently leading to **oxidative stress** which disrupts cellular homeostasis. (Moura and Memili, 2016)

Secondly, cryopreservation interrupts the mRNA content, which the sperm is incapable of replacing. This is critical because the spermatozoon releases mRNA in the oocyte, which plays a role in the early embryonic development stages, hence affecting the fertilizing capacity.

There is also a link between **DNA methylation** and conception rates; methylation involves the addition of a methyl group to cytosine guanine regions (CpG or 5'-C-phosphate-guanine-3') via covalent bonds. This is a critical process for embryo development prior to implantation. Studies have demonstrated that cryopreservation stress affects the degree of DNA methylation. The level of methylation in horses increases significantly post cryopreservation, unlike in ruminants and swine. (Aurich et al., 2016) This was confirmed by Aurich et al in 2016, where a group of good freezer stallions with excellent post freezing quality (motility and membrane integrity) were assessed for methylation levels with and without cryopreservation using ELISA. Raw semen samples and shock frozen samples exhibited low DNA methylation, in contrast to significantly high methylation levels ($P < 0.05$) in cryopreserved samples. In addition, a variation between individual stallions used in the study was noted in regards to the level of hypermethylation, but no link between age groups (stallions in the study were aged between 8 and 23 years old).

The abnormal methylation in frozen-thawed semen is believed to interrupt embryo development and pre-implantation, hence being a factor contributing to poor fertilization rates. However, it has not been discerned whether it is hypermethylation or hypomethylation that is responsible for poor fertilization rates. Since such studies, it has been suggested to include DNA methylation as an additional parameter for the evaluation of sperm quality.

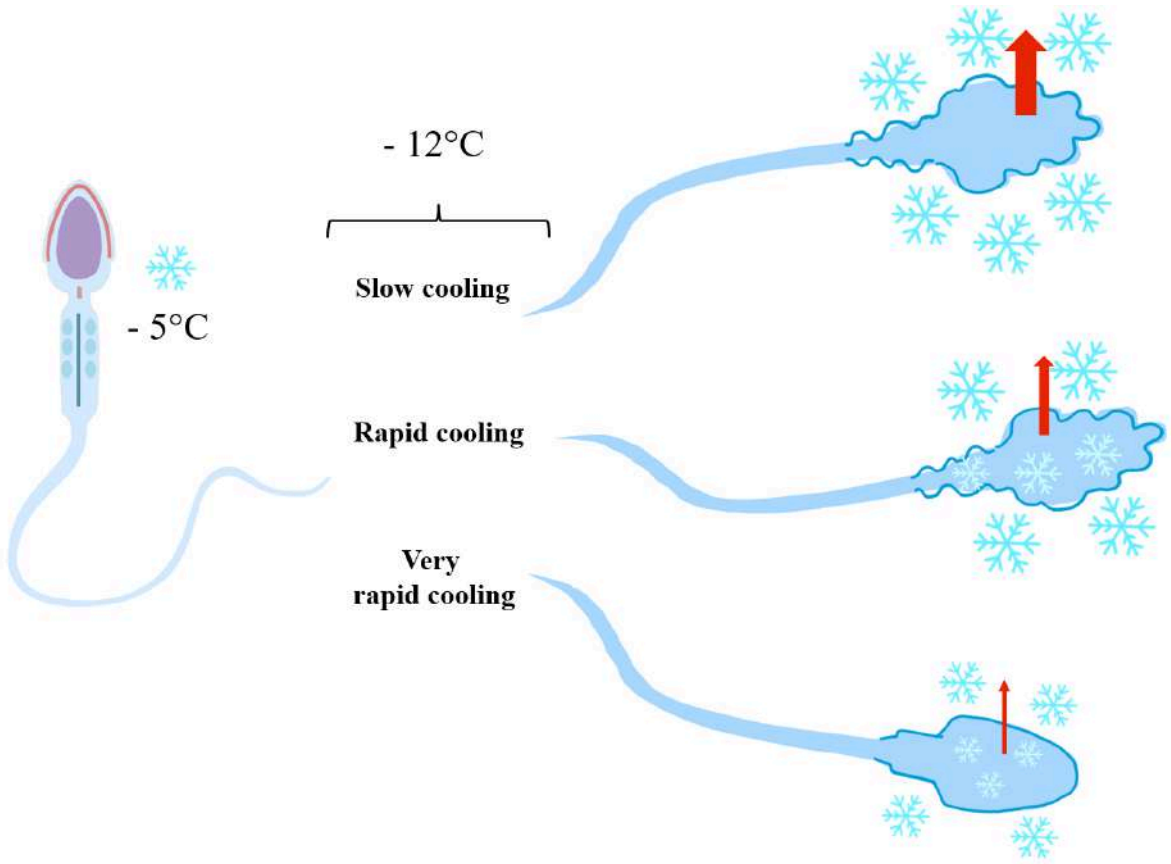


Fig 3.1 Physiological effects of cooling and freezing rates on spermatozoa physiology.
(Illustration by Cristina Martinez, 2024)

4. Advances & Developments in Cryopreservation Protocols

Extensive research over time has been able to pinpoint the origin and main sites of sperm cryoinjury. This has provided valuable information for scientists to reformulate protocols and introduce new approaches to diminish the deleterious consequences of cryopreservations, with the goal of improving fertility rates while being able to utilize the benefits of frozen semen.

Much research has been focused on countering cold shock by improving the cooling, freezing and thawing rates with more controlled temperature protocols and implementation of freezability markers; as well as by investigating the most effective types, concentrations and combinations of CPA (CPA) (Medeiros et al., 2002)

Cushioned Centrifugation

The speed and duration used for centrifugation varies between individual stallions; using a centrifugation cushion simplifies this process and uniformly centrifuges individual semen. This was developed to enhance the individual tolerance to centrifugation. The outcomes of using a cushion have been very successful, with sperm recovery rates of 90-95%, hence it has become a popular method. (Sieme et al., 2003) The application of the cushion maximizes the quality of the semen sample, as it lessens centrifugation damages, even though it implements high centrifugation forces. The cushion medium consists of a non-ionic iodinated compound which is often Ioxidanol. This medium is also utilized as a density gradient for those individuals with low sperm concentrations. (Bliss et al., 2012)

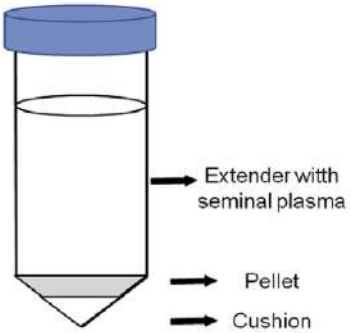


Fig. 4. Schematic representation of the cushion centrifugation. (Alvarenga et al., 2016)

The steps of this technique are as follows (Alvarenga et al., 2016):

1. Semen sample is diluted with a skim milk based extender in a 1:1 ratio, into a 50 ml tube.
2. Cushion fluid (1-5 ml) is carefully layered underneath the extended semen sample, at the bottom of the tube using a catheter and syringe. The sperm layer will immediately form above this.
3. The sample is centrifuge at 1000 x g for 20 minutes.
4. Afterwards, the supernatant (seminal plasma or the extender) is removed with care using a catheter and syringe or vacuum pump.
5. Next, the cushion fluid can be aspirated out with a catheter if necessary; although it is sometimes recommended to leave behind 5-10% of seminal plasma to aid sperm motility in the uterus and subsidize any inflammatory responses in the mare.
6. The pellet is ready to be resuspended in the desired freezing extender.

Some cushion fluids available include: Eqcellsire (France), Cushion Fluid (Minitube, Germany), and Red Cushion (Botupharma, Botucatu, Brazil).

Density Gradients

Commercial density gradients like EquiPure and Androcoll-E (Minitube) have been introduced to select out the spermatozoa affected by impaired motility, integrity and morphology. This is especially useful if the initial semen quality was undesirable, and the density gradient is applied before freezing. Sperm may also be selected post-thawing, by layering the content of 4 straws over 2 ml of EquiPure and centrifuging at 300g for 20 mins. The pellet is resuspended with the freezing extender. Dead or poor quality sperm produce oxidative reagents, therefore removing them has a benefit on the fertility success. (Alvarenga et al., 2016)

Freezability markers

Sperm proteins maintain cellular integrity, morphology, and play an important role in motility, acrosome reaction, capacitation, fertilization, thus influencing the success of fertilization and embryo development. Additionally, proteins found in the seminal plasma protect sperm cells in the epididymis and in the female reproductive tract. (Moura and Memili, 2016) Freezability markers are proteins associated with the sperm cell's ability to survive cryopreservation stress (Yeste, 2015). (Yánez-Ortiz et al., 2022)

Recently, proteomics has become more available to provide valuable information about sperm cryo-tolerance and sensitivity via protein markers. (Ďuračka et al., 2023) This helps us improve and tailor freezing protocols to the individual needs of the stallion's semen, especially for those classified as poor freezers. (Ďuračka et al., 2023) For instance, A-kinase anchor protein 4 (AKAP4) and its precursors are used as markers to assess sperm quality and thus the extent of cryo damage in pigs, horses and sheep. Similarly, heat shock proteins HSP90AA1 and HSPA8 are linked to cryotolerance; they are found in greater amounts when the resilience to freezing is higher. This was proven by Casas et al in 2010 when they found that swine sperm with high concentrations of HSP90AA1 possessed had higher survivability rates to cryopreservation. The resilience to freezing and thawing was assessed by seme post-thawing motility and viability. (Yánez-Ortiz et al., 2022) These proteins play a role in the sperm's survivability after thawing, as they are linked to the sperm's ability to withstand exaggerated oxidative stress, and are associated with the changes in the protamine-DNA complexes, which occur during freezing. (Yánez-Ortiz et al., 2022)

Test Freeze Procedure

Individual differences between stallions in regards to sperm viability, does not make it possible for a single universal cryopreservation method to be used. Because of this, the viability of the semen sample from each individual must be evaluated post cryopreservation. The test freeze procedure was developed to help determine whether an individual stallion is able to produce sufficient post-thaw quality semen, after altering certain steps of the cryopreservation protocol. The protocols differed based on the centrifugation technique, extender composition, cooling rates and packing. After each experiment, the semen samples are analyzed.

In 2007, Loomis and Graham assessed the split test freeze procedure in 224 stallions, by evaluating the total sperm number, progressive motility, sperm morphology and bacteriology. Prior to freezing, the sperm quality was evaluated using CASA.

The steps of their test freeze procedure involved:

1. Dilution of the semen sample in an extender composed of skim-milk and glucose.
2. The sample is centrifuged to separate the concentrated sperm cells and seminal plasma.
3. The concentrated sperm cell fraction is re-suspended into four different extenders. The extenders differed in the amount of sugar and salt (A), amount of milk (B), egg yolk (C), buffer type and amount of cryoprotectant (D).
4. The semen and extender sample is poured into 0.5 ml straws, then cooled and frozen in a controlled freezer.
5. The straws are then stored in liquid nitrogen for over 24 hours
6. Thawing and evaluation takes place (thawing temperature was not indicated in the paper).
7. The thawed semen sample is cultured for bacterial pathogens.
8. A fraction of the thawed sample is diluted in skim milk-glucose extender, incubated at 37°C for 30 min.
9. CASA was used to evaluate the post-thaw motility. A sperm is motile if it has an average path velocity (VAP) above 50 m/s. Non-motile sperm velocity is below 20m/s.

In this test freeze study, 85.3% of stallions (191 out of the 224 individuals) produced semen with post-thaw progressive motility above 30%. Loomis discovered that protocol D yielded the best post-thaw progressive motility for each individual stallion (58%). Protocol A (different sugar and salt concentrations) had the poorest motility results (3%). The methods using different milk (B) and egg

yolk (C) concentrations resulted in 11% and 28% post-thaw progressive motility. This may suggest that the amount of buffer, cryoprotectant, and egg yolk, are the factors of most importance when aiming to improve post-thaw motility. A limitation to this experiment is that it excluded the effect of different temperatures and exposure time throughout cooling, freezing and liquid nitrogen, as well as the centrifugation rate.

Contrary to an experiment by Sampler et al 1994, which showed no significant correlation between individual stallions and type of extender used. On the other hand, research conducted in France by Ecot et al in 2000, found that the post-thaw semen quality was significantly different based on the cooling method and the type of extender used. Despite this, interaction between extender type and the individual stallion was not consistent.

Semen Extenders & Cryoprotectants: Purpose & Function

For cryopreserved sperm to maintain their function and cellular metabolism, it is important that their milieu is kept undisturbed. Energy, pH, osmolality, ionic concentrations are the main factors necessary for sperm to withstand temperature and structural changes that come with cryopreservation. Semen extenders were developed to conserve the vital properties of spermatozoa throughout cryopreservation, and therefore aid fertilization success. They provide nutrients and energy (ATP) to maintain cellular metabolism, they also have buffering properties that stabilize pH, osmolality; neutralize toxic metabolic by-products and reduce oxidative stress with its antioxidant features; finally, extenders protect the plasma membrane from thermal and oxidative stress of cooling and freezing.

A **primary extender** is used to dilute the raw semen sample before centrifugation. These are typically buffered saline or buffered sugars, such as glucose-EDTA, or skim milk solutions, like INRA-82. Often, skimmed milk or casein based extenders are used for stallion semen. The purpose of this is to preserve sperm activity after it is separated from the seminal plasma. (Alvarenga et al., 2016)

Freezing extenders contain a cryoprotectant which has several protective roles throughout cryopreservation. CPAs prevent intracellular and extracellular ice crystal formation, minimize exposure and damage caused by osmotic stress, and reduce the effects of ROS (Harald Sieme, et. al. 2015)

CPA can be penetrating or non penetrating, and may have an intracellular or extracellular function. Intracellular CPAs bind water, while extracellular agents create a hypertonic environment to stimulate diffusion and dehydrate the sperm cell enough which would reduce intracellular ice crystal formation. **Non-permeating CPAs** are protective during freezing; these include egg yolk, milk and certain sugars. (Alvarenga et al., 2016) It is important to note that **permeating CPA** can have toxic effects at certain concentrations, hindering cell membranes and sperm motility. (Medeiros et al., 2002)

Semen extenders used for freezing contain cryoprotectants made of skim milk, egg yolk, or glycerol. The first major advancement that was made half a century ago, on fowl and bull sperm, was the discovery of egg yolk and glycerol for cooling and freezing; this was further investigated on other species of agricultural importance. (Medeiros et al., 2002)

There is a variety of commercial extenders on the market composed of different CPAs; some examples include INRA 96 containing caseinate, BotuCrio, Gent A containing egg yolk; others often have a concentration of glycerol, or a combination of glycerol and other ingredients like egg yolk or milk, etc. (Bustani & Baiee, 2021) A combination of CPA provides more efficient cryo-protection rather than using a single CPA.

The general media used in semen cryopreservation for the majority of species contains a buffer, a non-permeable cryoprotectant, a permeable cryoprotectant, an energy source which usually being glucose, and other additives such as antibiotics, vitamins, and antioxidants. The most common antibiotics added include procaine penicillin, ampicillin, gentamicin sulfate, lincomycin, etc. (Pesch & Hoffmann, 2007) Most commercial freezing media contain approximately 20% of egg yolk (non-permeable) and 3 to 5% of glycerol (permeable). Additionally, studies have been able to prove the benefits of supplementing the semen sample with cholesterol to counteract the harmful exposure to cold shock. (Yánez-Ortiz et al., 2022)

Egg yolk & Milk

Egg yolk has been utilized for over 65 years as an extender to protect mammalian semen. Its protective mechanism on the sperm membrane is not fully understood, although it is believed that the low-density lipoproteins (LDL) and phospholipids content plays a protective role against cold shock. These lipoproteins bind to the cell membrane and replace the phospholipids that have been lost during freezing; hence, this stabilizes and protects the lipid bilayer. (Alvarenga et al., 2016)

Additionally, the phospholipids in the egg yolk may reduce cellular injury caused by cold stress. (Medeiros et al., 2002) Optidyl, Triladyl or Gent A are examples of egg yolk extenders. (Bustani & Baiee, 2021) Despite this, using egg yolk poses the risk of potential bacterial contamination, especially due to the high prevalence of salmonella in laying hens (Pesch & Hoffmann, 2007). Because of this, scientists are currently investigating plant-origin phospholipid substitutes from soy lecithin, as it functions similarly to LDL. Moreover, LDL can be used on its own if isolated from the egg yolk; doing this, has proven to yield better motility and viability post-thawing in horse, cattle, pigs, and sheep. (Yánez-Ortiz et al., 2022)

Milk is believed to have cryoprotective function due to its high protein content, which buffers the semen pH and chelates heavy metal ions. Furthermore, lactose in the milk cannot bypass sperm membrane since it is hydrophilic, therefore protecting the cell wall from freeze shock.
It has been used in combination with arabinose, fructose or egg yolk. Some examples of commercial extenders containing skim milk are BotuSemen and Equiplus. (Bustani & Baiee, 2021)

Glycerol

This is the most universal, conventional CPA, utilized for many years. This is a permeable cryoprotectant that penetrates the cell membrane. It enhances the sperm cells resistance to freezing by altering membrane diffusion rates, thus creating a tolerable osmotic concentration for the sperm. It has an extracellular effect which involves a rapid diffusion of water out of the cell; this therefore osmotically leads to dehydration to avoid freezing of intracellular water and thus formation of crystals. (Yánez-Ortiz et al., 2022) Glycerol reduces intracellular osmotic stress caused by dehydration, by replacing the intracellular water and lowering its freezing point.

Despite glycerol's protective features from cryodamage, it has a contraceptive effect on fertilization rates. Addition and removal of glycerol (when it permeates into the cell before freezing and out of the cell at thawing) is when it induces an osmotic stress, especially if diffusion rates are too rapid (Alvarenga et al. 2005) This often hinders post-thaw motility and viability, although studies have proven reduced fertility in stallion, boar, and ram semen cryopreserved with glycerol, regardless of acceptable progressive motility post-thawing. (Amann and Pickett, 1987). The reason for this may be that glycerol when glycerol binds to the phospholipid heads on the membrane, it directly reduces

membrane flexibility. These adverse effects may be avoided by eliminating glycerol from the insemination dose, using a stepwise dilution that draws out the glycerol molecules.

A study conducted by Burns and Reasner in 1995 on the effect of different glycerol concentrations (0, 1, 2, 4%) on stallion sperm, demonstrated that lower glycerol concentrations were more beneficial. Velocity and progressive velocity had the best results at 1%. On the other hand, concentrations over 2% revealed no improvement on progressive motility and lateral head displacement. Semen added to a medium of 2.5% glycerol had significantly reduced velocity, possessed lateral head displacement and a greater number of sperm had abnormally wrinkled acrosome membranes. Nonetheless, no change was observed in the motility. (Burns and Reasner, 1995)

Based on the results by Macías Garcías et al 2012, concentrations should not exceed 2.5% when freezing stallion semen, as glycerol becomes toxic at concentrations ≥ 3.5%, exerting the highest level of toxicity at 5%. Some individual stallions exhibit higher sensitivity to glycerol than others.

High concentrations inflict osmotic damage to the spermatozoa membrane; it increases the osmotic pressure of the diluent, dehydrating the sperm cell which leads to protein denaturation. In the aftermath, the plasma membrane morphology, acrosome integrity and function is hindered. By adjusting the osmotic pressure of the diluent we can find the optimal glycerol level that causes the least damage while still retaining its protective role. (Macías García et al., 2012)

Cholesterol

As discussed, one of the challenges of sperm cryosurvival is the alteration in the cell membrane conformation post-cooling, caused by a loss of cholesterol in the membrane. It was recognized by Ladbrooke et al 1968, that higher proportions of cholesterol to phospholipid in the cell membrane prevents a shift from liquid to a gel state during cooling. (Loomis and Graham, 2008). Moore, et al 2005, also advised that in order to prevent cold shock, the membrane must remain stable and a fluid to gel transition should be avoided. Furthermore, cholesterol increases the osmotic tolerance of stallion and donkey semen. Oliveira et al., 2010, proved in their study on stallion semen, that cholesterol protects the plasma membrane against the damaging effects of freezing. Moore explored the effect of different cholesterol concentrations on sperm cryosurvival, by assessing what concentration of Cholesterol Loaded Cyclodextrin (CLC) generated the best survival rates. Data revealed that the sperm cells continued to possess high levels of cholesterol after CLC was supplemented at the start of

cryopreservation. Behera et al in 2015 treated 48 ejaculates of sexually active Malbri bucks with cholesterol, and found significant improvement in pre-freeze and post-that progressive motility.
These results suggest that high cholesterol concentrations increase the percentage of viable sperm post-thawing, since it reduces the risk of cells experiencing cold shock and therefore yielding a higher number of sperm with intact plasma membrane integrity post-thawing. Accordingly, the post thaw semen quality is greater, which has positive outcomes regarding the number of sperm capable of binding to the ZP (Moore et al., 2005). There is a variation of cold-shock susceptibility between different species because of the different cholesterol to phospholipid ratios. Human and rabbit sperm possess high proportions of cholesterol to phospholipid ratio, which is arguably the reason as to why their sperm cells are more resistant to cryoinjury. (Amann and Pickett, 1987) Equine sperm have a 0.36 ratio of cholesterol to phospholipid in their plasma membrane, which is intermediate to boar and bull sperm. (Oliveira et al., 2010)

Despite these benefits, excess CH impairs the flexibility of the stallion spermatozoa, as it makes the plasma membrane rigid like. This is negatively reflected on the in vivo fertility rates: the pregnancy rate of mares inseminated with cholesterol supplemented semen was 25%, in contrast to 75% in mares inseminated with semen that was not incubated with cholesterol. By altering the rigidity of the membrane, CH indirectly blocks the acrosome reaction. A rigid plasma membrane has reduced permeability to calcium which means the adenyl cyclase activity won't be activated in order to stimulate capacitation and acrosome reaction (Oliveira et al., 2010). Studies have therefore been carried out to investigate the removal of CH from frozen-thawed spermatozoa with cyclodextrins.

Use of Cyclodextrins

CD are water-soluble compounds that harbor lipids within its molecule. Methyl-β- cyclodextrin (MβCD) is a cyclic oligosaccharide with a hydrophobic center that has high affinity to steroids, including cholesterol. Water molecules are trapped in the cyclodextrin center, however when in contact with the plasma membrane, they uptake the cholesterol (lower polarity) into the cyclodextrin cavity, and expel the water molecules.

Oliveira et al. also studied the effect of MβCD on cholesterol treated stallion semen during thawing. The groups in the study incubated with MβCD were not significantly different in regards to their acrosome reaction, despite using different MβCD concentrations between the two groups. Overall, the sample groups treated with MβCD had an increased proportion of acrosome reacted sperm, unlike in the untreated group (see figure 5.). Other experiments conducted on equine and bovine semen, found that the post thaw motility increased when MβCD was added during thawing.

Once cholesterol is eliminated post-thawing, capacitation and acrosome reaction is stimulated, which restores the fertilization capability of the stallion sperm. The number of sperm that has undergone an acrosome reaction can be quantified by the percentage of sperm with an intact membrane after induction. (Oliveira et al., 2010)

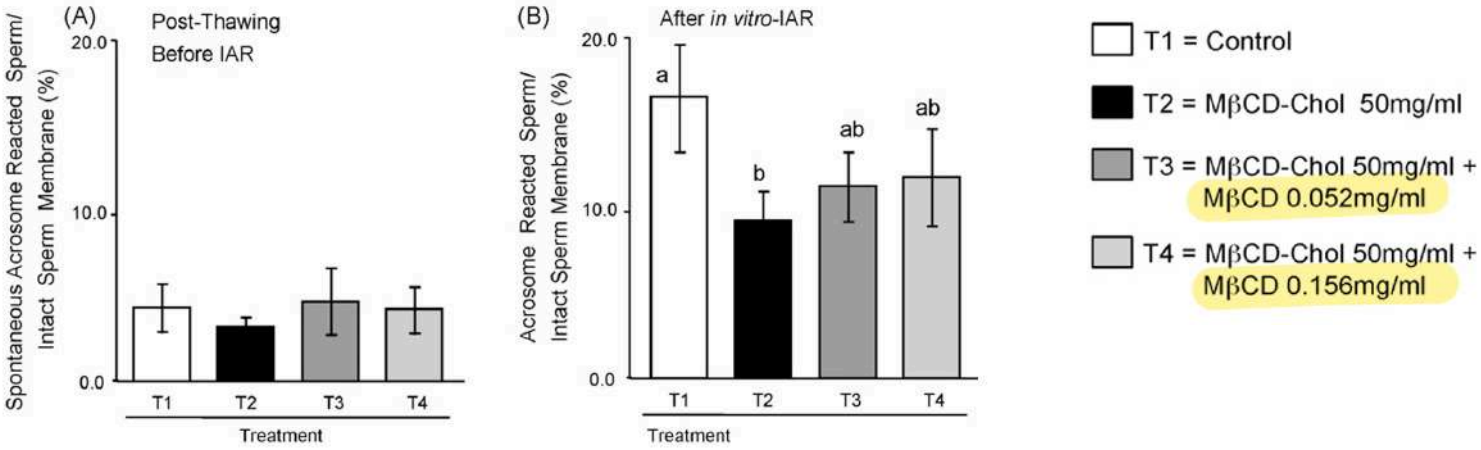


Fig. 5 Mean percentage of frozen-thawed stallion spermatozoa that have undergone a spontaneous acrosome reaction (A) and percentage of spermatozoa with induced acrosome reaction (B) (Oliveira et al., 2010)

The same concentration of cyclodextrin (CLC) used to incorporate the cholesterol into the membrane is not sufficient to remove it. However it is vital to consider the concentration of MβCD used to remove cholesterol, because too high concentrations have damaging effects on the physical integrity of the sperm and thus its life span. (Oliveira et al., 2010)

5. Limitations & challenges of stallion semen cryopreservation:

Although a lot of effort has gone into perfecting cryopreservation protocols, approximately 50% of the originally motile sperm population does not survive thawing. Because frozen-thawed sperm have reduced survivability, only a smaller percentage of sperm possess the ability to bind to oviductal epithelial cells (OEC). Nonetheless, this can be overcome by increasing sperm population in the insemination dose or by depositing the semen closer to the site of fertilization and carefully timing the ovulation.

Factors affecting semen quality and cryopreservation success

Throughout each phase of cryopreservation, spermatozoa are met with cellular damage of varying degrees of severity. The accumulation of injuries are reflected upon the functional state of the post-thawed sperm cells. Repercussions of cold shock include: irreversible motility loss post thawing, reduced selectivity of the membrane permeability and damaged acrosomal membrane. (Medeiros et al., 2002)

The impaired sperm membrane permeability to water and ions, causes abnormal metabolic function, motility loss and premature cell death. These damages are similar to those effects that occur post capacitation; both processes reduce function and viability of spermatozoa in the uterus.

Individual variability in semen quality

Variations in sensitivity to the adverse effects of cooling exist between different species due to the different membrane structures. Boar, bull, ram, stallion sperm are noted as the most sensitive; dog and cat sperm are relatively sensitive; while rabbit, human, and rooster have the least sensitive sperm to cold shock (Medeiros et al., 2002) Boar sperm is highly susceptible to cold shock, that is why chilled semen is preferred method for AI in swine. Despite species specific protocols, the fertility rates of frozen sperm still remain lower than those achieved with fresh semen samples.

There is a discrepancy between individual horses in regards to sperm survivability following cryopreservation, as each stallion presents a unique tolerance to thermal and osmotic stress. Semen quality differs among sires, ejaculates, and even between fractions of the same ejaculate sample. (Yeste, 2016)

The concentration of certain proteins (freezability markers) can inform us about the sperm's survival rate; the concentration of these proteins varies between stallions. (Moura and Memili, 2016)

The equine breeding industry suffers more challenges compared to the dairy industry. Bull semen is selected depending on its ability to survive cryodamage; hence, cattle have a more uniform outcome regarding conception rates. On the other hand, stallions are bred for desirable genotypes; thus, in order to overcome such individual variations, cryopreservation protocols must be tailored, so that we can obtain more positive results. (Loomis and Graham, 2008)

Influence of Stallion Age

Studies carried out in Vienna Vetmeduni, 2020 by Jörg Aurich et al. found that stallion age was an important factor influencing the semen quality. Stallions reach full spermatogenesis capacity after the age of six years. The post-thaw motility is lower in stallions below six years of age, in comparison to older stallions. Much elderly stallions of course, tend to be subfertile with lower semen quality. Stallions over the age of 9 years had reduced percentages of acceptable cryopreserved ejaculates. Freezing protocols may also be adjusted accordingly based on the age, in order to continue achieving relatively high quality sperm banks for the breeding season. In addition, the composition of the seminal plasma, which determines whether the semen is suitable for freezing, also varies between age groups and breeds. (Aurich et al., 2020)

Influence of Stallion Breed

Horse breeds also play a role in the acceptable quality of cryopreserved semen: Arab stallions have superior quality ejaculates, while Icelandic and Quarter Horse stallions have a lower quality. Warmblood and Lipizzaner stallions were close to the average. The raw parameters of these breeds varied (volume, sperm concentration, and total sperm count), however, there are no significant differences in sperm motility among breeds, but minor differences have been reported in some cases.

6. Differences in Cryopreservation Protocols

Semen cryopreservation protocol: Vetmeduni Vienna

For semen collection, a Hannover artificial vagina is used along with a dummy and the presence of a teaser mare. The gel fraction is removed after collection and the semen is filtered through a gauze. The ejaculate sample is then examined for volume and color, the sperm concentration is determined using SpermaCue, and the total motility and membrane intact spermatozoa are identified using CASA, as mentioned previously. The orientation change, curvilinear velocity, and the distance in a straight line are examined with specific acceptable thresholds, to determine the overall motility.

The sperm membrane integrity is examined as follows: a semen sample of 100 µL is mixed with 2 µL of propidium iodide and incubated for 10 min at room temperature in darkness. A droplet is then evaluated by fluorescence microscopy. When evaluating the results, the structures stained green are viable spermatozoa heads, indicating that the membrane is intact. On the contrary, those spermatozoa stained in red have damaged membranes; see figure 6 . (Aurich et al., 2020)

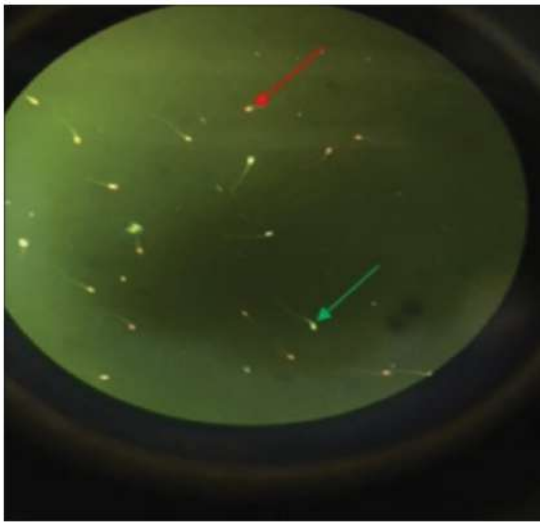


Fig. 6 Viable spermatozoa are stained green (green arrow), while dead spermatozoa stain red appear green (red arrow). Photograph by Alamaary et al., 2019

The following are the steps of the standard protocol used in Vetmeduni vienna:

1. The semen sample is diluted 1:1 with EquiPlus extender (Minitube).
2. Next the sample is centrifuged at 700 g for 12 min, and the resulting supernatant aspirated.
3. The remaining purified sperm sample is mixed at a ratio of 1:1 with Ghent freezing extender (Minitube).
4. A concentration of 2.5% glycerol was added to the extended semen.
5. Finally, the semen is placed into 0.5-mL straws which are sealed automatically at room temperature.
6. The straws are then set on a rack in a freezing chamber with a controlled temperature of 5°C with a cooling rate of 0.3°C/min. Then the freezing gradually begins within 3 min, firstly to −25°C (10°C/min), and then to −140°C at a rate of 25°C/min.

7. The straws were removed from the freezing chamber and directly plunged into liquid nitrogen where they are stored for 24 h before thawing.
8. One straw from each ejaculate is taken out for thawing to determine the post-thawing characteristics of the spermatozoa (progressive motility and sperm membrane integrity). For thawing, the straw is placed in a 37°C water bath for 15 seconds.

This study done in vienna followed the European studbook recommendations, which classifies frozen-thawed semen as acceptable for insemination when the progressive motility is ≥35%.
The sperm motility in raw semen is considered the most important factor influencing the classification of ejaculates as acceptable post the cryopreservation process.

Semen Cryopreservation Protocol: University of Hannover.

Experts at the university of University of Hannover (Herald Sieme, et al. 2015) defined the optimal cooling rate for semen freezing as: the rate at which the damage caused by intracellular ice formation due to rapid cooling, and osmotic dehydration caused by slow cooling, is kept to a minimum.
After addition of the freezing extender, Herald Sieme et al. suggests that the most adequate cooling technique is a slow and cautious temperature change from room temperature to 5°C. These steady methods minimize risks of cold shock therefore avoiding irreversible morphological changes to the membrane. Following the slow cooling, semen is packed into straws of 0.5 ml, while still maintaining the temperature at 5°C. The suggested optimal cooling rate for sperm is 40-60 °C min, which can be achieved by placing the straws 2.5-5 cm above liquid nitrogen for 20 minutes. Finally, they are plunged into and stored in liquid nitrogen. When needed, the thawing process is quick, involving incubation of the straws for 30 seconds at a temperature of 37ºC.

Semen cryopreservation Protocol: Al Hawajer, Sharja, UAE

I had the opportunity to observe and help veterinarians at Al Hawajer Stud farm, UAE, carry out their artificial insemination protocol, which is summarized as follows:

1. Firstly, the stallion is prepared for semen collection by washing the penis and urethral fossa using warm water. The goal is to minimize the contamination of microorganisms found in the smegma. Following the traditional methods, a phantom is prepared and the mare in heat is brought into the semen collection center.

2. The modified Colorado artificial vagina is prepared by Dr. Frederico Freitas (figure 2.3), by placing a plastic interior lining and filling the AV with hot water (approximately 40 - 50 °C). The use of lubricating gel was avoided in this farm, to avoid any risk of lubricant contents damaging the sperm viability.
3. The semen sample is filtered into a flask (Figure 4.1) in order to filter out the gel portion, impurities or debris, and to obtain the desired semen sample.
4. A small sample is collected from the total filtered sample using a pipette and is placed in a spectrophotometer to determine the semen concentration. (Figure 4.2 and 4.3) This helps us calculate an approximate number of straws needed.
5. Another small sample from the pipette is used for microscopic examination of motility and viability.
6. The complete filtered semen sample is then mixed with a diluent to a 1:1 ratio (5 ml of diluent is added to 5ml of the semen sample. The diluent or semen extender used is INRA 96, which is composed of milk but chemically optimized for sperm survival and storage.
7. Dr. Frederico sets the centrifugation speed to 800g / 20 mins, as he found an increased risk of damaged sperm cells if the speed is accelerated to 1000g. The goal of centrifugation is to separate the diluent and purified sperm samples. (Figure 4.4)
8. Afterwards, the supernatant diluent layer is carefully removed using a pipette and discarded, so that we are left solely with a pure sperm sample. (Figure 4.5)
9. Next, 10 ml of an egg yolk based extender is used as the preservation method. (Figure 4.6). The whole sample along with the extender are injected into individual straws. (Figure 4.7)
10. Finally, the cooling process begins with the straw samples placed in a fridge with controlled temperature at 5 °C for 20 minutes.
11. The sample is then placed solely in nitrogen vapor for 20 minutes, which is followed by plunging the straws into the liquid nitrogen, and then being stored. (Figure 4.8 and 4.9)

All the minor detailed steps during semen collection, thawing and freezing, play an important role in the resulting sperm quality, including the quality of the materials and lubricants used.

Fig. 7. Semen collection

Fig. 7.1 semen sample is filtered into a flask

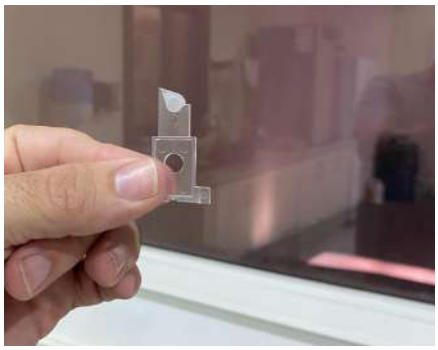


Fig. 7.2. Small sample of the semen to be placed in the spectrophotometer.

Fig. 7.3 Using a spectrophotometer to determine the semen concentration

Fig 7.4.1 centrifugation of the sperm sample following the addition of the diluent

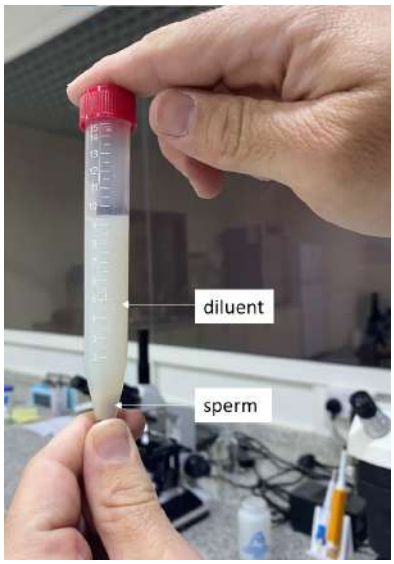


Fig. 7.4.2 Separated layers of the diluent and sperm sample.

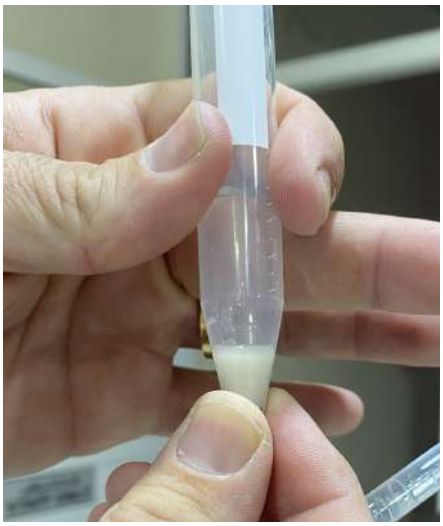


Fig. 7.5 pure semen sample collected after removing the separated diluent layer

Fig 7.6 Egg yolk base extender is used for the preservation of the sample.

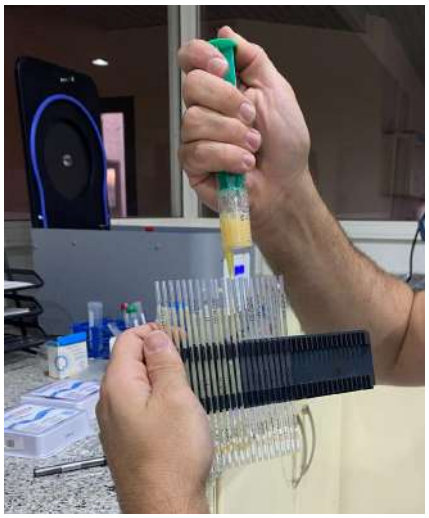


Fig 7.7 Injecting the mix of semen sample with egg yolk extender into straws which are sealed and closed.

Fig. 7.7.1

Fig 7.8 Cooling process. Samples are placed in the nitrogen vapor and then plunged into liquid nitrogen for storage.

All images (Fig. 7 - 7.8) taken by Cristina Martinez April 2023, Al Hawajer, Sharja, UAE

6. Insemination Doses with Frozen Semen

An insemination dose may involve 1, 2, or 8 straws or a combination; this is dependent on the motility. When multiple straws are used per breeding dose, they are usually 0.5 ml. The insemination method applied also depends on the number of doses available. (Dascanio & Kasimanickam, 2008) The success of frozen semen insemination depends on the insemination technique used, and what strategies are combined, such as estrus synchronization, induction of ovulation, careful insemination timing, and deep horn insemination. Veterinarians at Wisconsin Equine Clinic and Hospital found that accurate timing of a deep horn insemination can provide successful pregnancy rates, even with low doses. Throughout four of their seasons, general insemination with frozen semen resulted in an overall fertility rate of 46% for all cycles (629), while insemination with half a dose of frozen semen yielded pregnancy rates of 49% (178 out of 364 cycles) (Schmidt, 2011). However, despite the successful fertility rates, it was concluded that the semen quality and accurate timing of insemination are far more important than the number of spermatozoa. Additionally, purchasing semen from well known stallions is costly, so dividing the dose allows for storing semen for another insemination cycle, and also reduces the risk of post-breeding endometritis.

Using one insemination dose is the least desirable case, as it emphasizes predicting the ovulation time, to either inseminate prior or after ovulation. This also becomes expensive for the owner. If only 1 dose is being used, deslorelin is administered when uterine edema and a 35 mm follicle has been detected.

With 2 doses, one insemination is done before the ovulation estimate and the second dose is inseminated post ovulation. (Dascanio & Kasimanickam, 2008)

7. Insemination protocols with frozen semen

There are a few techniques applied in practice to inseminate mares with frozen semen, each having its own unique benefit. In general, it is acknowledged that the best window to obtain for insemination is no more than 12 hours before, and no more than 6 hours after ovulation (Alvarenga et al., 2016). Some protocols suggest daily evaluation and monitoring during estrus, by rectal palpation and ultrasonography every 6 hours; however this may be impractical for the veterinarian and expensive for the owner. (Austin, C. 2020) When a follicle measuring over 35 mm in size is detected along with uterine edema, ovulation can be induced either with human chorionic gonadotropin (hCG) (1500 IU)

or gonadotropin-releasing hormone (GnRH, deslorelin 1 mg). Once induced, Dr Alvarenga et al, 2016, recommends ultrasound evaluation every 6 hours until ovulation is detected. However, the mare may also be inseminated at fixed hours (ie. at 24 and 40 hours or 30 and 48 hours after ovulation induction)
The AI technique of choice depends on factors like the reproductive history of the mare, semen quality, the available resources and experience of the staff. It is common for veterinarians to stick to one technique or use two techniques parallelly.

1. **Conventional post ovulation protocol:**

The follicle is examined daily until it reaches a size of 35 mm. Injection with hCG or GnRH is optional. Rectal palpations should be performed every 6 hours in order to determine whether ovulation has occurred. The mare is inseminated 6 hours after ovulation has been detected. This is often done in situations where the semen source is either limited, expensive, or of low quality. The disadvantage of this method is the labor intensive frequent rectal palpations. (Mottershead, J. 2021)

2. **Pre and post ovulation protocol**

The mare is examined daily until the follicle reaches a size of 35 mm or larger, followed by administering an injection of hCG or GnRH. half the dose is inseminated 24 hours following the injection and the mare is then palpated daily. The next half insemination dose is administered 6 hours after ovulation has taken place.
A study conducted in Italy (Barbacini S, 2000) suggested that the suitable dose of hCG should be around 2,5000 i.u. For a mare that is approximately 630 kg, as an overdose and prevent ovulation.

3. **Estrus detection and Timed insemination**

This technique relies on accurate monitoring and induction of ovulation and a sufficient supply of semen. Similarly, the mare is examined until a follicle of 35mm is present, then hCG or GnRH injection is administered. This protocol involves insemination with one dose, 24 hours after the injection of the post ovulating agent, and the next insemination dose is given 40 hours after.
Previous research has indicated that 80% of mares ovulate within 48 hours after the hCG dose. This technique allows for better control of insemination timing. However, frequent monitoring comes with increased costs, as well as hormonal treatments, which are a disadvantage. Additionally, some mares

with reproductive conditions are not suited to receive hormonal stimulatory drugs, or in general may not respond to them, thus this protocol does not provide a hundred percent accuracy.

4. **Deep uterine insemination:**

Doses containing lower sperm counts are specifically administered into the uterotubal junction. This method has been shown to increase conception rates even with low doses or subfertile semen. Interestingly, lower conception rates occur with this method when high, good quality semen is used. Moreover, this technique may be used in conjunction with either one of the above mentioned insemination protocols. The biggest benefit of this method is that it conserves semen, (Austin, C. 2020) while also targeting the site of fertilization, thus maximizing the chances of a successful conception rate. The drawback of this protocol involves the need of specialized equipment and trained staff, as incorrect placement reduces the conception rates.

8. Advantages and Disadvantages of Cryopreservation

This section aims to evaluate the advantageous uses of cryopreservation, as well as the flaws that scientists are yet to overcome with this technology.

Advantages of Frozen Semen

1. Costs are reduced with frozen semen; multiple doses can be prepared and shipped at a much lower cost, in contrast to transporting the stallion or shipping one to two doses of cooled semen per breeding cycle. (Amann and Pickett, 1987)
2. Previously, stallions were either classified as show or breeding stallions, with shows occurring during springtime and overlapping with the natural breeding season. Stallions that were retired from competitions were switched to be used only for breeding. Now with the use of frozen semen, stallions are able to continue their competing career while also simultaneously being used for semen collection. This way, the stallion may continue to show off its performance abilities and reputation based on its genetics. (Skaife , 2013)
3. Another benefit that comes with utilizing frozen semen is the access to a global market. A bulk of doses can be shipped internationally for multiple breedings; the storage facility can then make domestic shipments when necessary, lowering shipping costs of a single international shipment. Not to mention, this also opens the opportunity to carry out inseminations regardless of the natural breeding seasons in different hemispheres; hence providing a source of income all year round. (Amann and Pickett, 1987)
4. Of course, unlike fresh and cooled semen, frozen semen can be stored long-term, and thus is available to the mare owner when necessary. This takes away the dependency on the availability of the stallion, and excludes the stress of hauling and transporting mares. Furthermore, this greatly reduces the risk of pregnancy wastage and exposure to pathogens in breeding farms. This also reduces breeding and insurance costs. (Amann and Pickett, 1987)
5. The semen of prized stallions can be stored for several years and used as an insurance policy when the horse has become ill or died, hence the owner can continue to sell breedings, excluding financial losses. Frozen semen can also replace unexpected issues with the quality of cooled semen shipments.

6. Frozen semen allows for a post-thaw analysis on the quality of the semen. Not only this, but an infectious disease screening can be done, to exclude venereal transmitted diseases like equine arteritis virus, equine infectious anemia, mycoplasma and Trypanosoma species.

Disadvantages of Frozen Semen

1. Cryo-injury affects the ability for sperm to bind to the oviductal epithelial cells (OEC) and to the ZP because of the affected membrane integrity and thus compromised motility post-thawing (spermatozoa attach to the OEC via their plasma membrane). (Dobrinski et al., 1995) The lifespan of frozen equine sperm in the female reproductive tract is also reduced, as opposed to fresh semen; this has been confirmed via in vitro studies. All these factors could explain the reduced fertility rates with frozen sperm.

   Spermatozoa attach to the OEC and pool at the isthmus of the oviduct; this attachment is meant to prolong the life span of sperm and thus its fertilizing ability, in vitro. As spermatozoa undergo maturation, they gain the ability to bind to the ZP in the oviduct, permitting the acrosome reaction to occur next. However, it has been reported that frozen-thawed semen has reduced motility in the isthmus and decreased binding capacity to the ZP.
2. In addition, functional differences exist in semen amidst individual stallions, which cannot be detected on conventional parameters (progressive motility, membrane and acrosome integrity); this was proven on competitive zona binding assay.
3. Pregnancy rates may be much lower when using frozen semen as opposed to fresh. Literature and research comparing fresh, cooled and frozen semen in equine breeding is relatively limited, moreover, the data regarding the use of frozen semen and its effect on conception rates is rather outdated. Amann and Pickett in 1987 suggested that only 25% of stallions will produce pregnancy rates that are similar to that of fresh semen or natural service, while 75% will have below optimal pregnancy rates (Amann and Pickett, 1987). Samper et al. in 1991 described that frozen semen can only result in an average of 30-40% pregnancy rate, with variations between stallions, plus considering the health of the mare. (Samper, 2001) Loomis in 2001 reported a 51% pregnancy success rate, however the most recent study in 2003 by E. Squires et al. demonstrated a 45% pregnancy rate. The most recent data, collected in 2010 from three breeding farms in Maryland USA, from a study that observed the outcomes of cooled and frozen semen from 36 stallsions and 648 mares; the results of the study found a 79%

conception rate with cooled semen, and 76% from frozen semen. Despite this data, commercially acceptable frozen semen can result in 50-55% conception rates in the first cycle and approximately 70-80% in seasonal pregnancy.

4. As seen in dairy farms, mobile freezing laboratory units produce suboptimal semen samples, despite its convenience. Hence, it is advised that stallions are transported to a central laboratory for semen collection and freezing, or that the breeding farm sets up its own processing laboratory.

Conclusion

Over the years, mare pregnancy rates have increased significantly and in recent years breeding registries take preference over frozen semen. Cryopreserved semen is more easily accessible, it can be transported through long distances, the risk of pathogen transmission can be omitted, and more importantly, it permits fast genetic gain. This stresses the importance for practitioners to be familiar with the challenges that may occur during processing, freezing and storing frozen semen. In addition, specialists should be aware of the variations that can occur in the semen quality from individual stallions, breeds and age groups, and how the standard protocol can be customized respectively, in order to maximize semen quality. However, this is not only limited to the individual stallions, but the tolerance to freezing varies between ejaculates of a singular stallion.

Although frozen semen has its own unique advantages, it still continues to be inferior to chilled semen regarding fertility rate success. A big reason for this is the fact that chilled sperm does not undergo nearly half of the damaging risks that frozen sperm do, therefore sperm in this chilled state is able to retain viability in the uterus for longer, compared to frozen-thawed sperm, which is directly proportional to healthy and successful pregnancies. (Bustani & Baiee, 2021)

The whole cryopreservation process from cooling to thawing predisposes the sperm cell to several stresses, which compromise the inherently fragile function and viability of the spermatozoa in the uterus, therefore negatively affecting fertility outcomes (Medeiros et al., 2002). Cryopreservation triggers thermal stress, oxidative stress, osmotic stress and much more, causing an overall irreversible disruption to the cell physiology and integrity. Capacitation, acrosome reaction and fertilization all rely on the intactness of the acrosome membrane. The disruptions of the acrosome directly prevents fertilization. (Bustani & Baiee, 2021) All these factors indirectly affect the ability of the spermatozoa to survive and accomplish its role in the uterus, hence why using frozen semen can be challenging. A lot of research has been carried out, focusing on the smallest details of sperm cellular function and morphology, in order to develop better protocols. Such advancements include the introduction of new or upgraded CPAs, extenders and freezing protocols, all aiming to reduce cryo injuries by preserving membrane integrity, which would in turn benefit the viability and function of the post-thaw semen. When utilizing good quality extenders, the fertility rates can be indirectly improved.

A scientific article written in the late 90s by James K. Graham suggests that fertility rates using frozen spermatozoa did not match the success of fresh semen inseminations. However, with the amount of experiments and detailed research on cryopreservation, significant improvements were accomplished, thus fertility rates with frozen semen have followed this success. (Graham, 1996)

According to Graham, with carefully executed standardized protocols, it is possible to achieve fertility rates of 40-60% for an individual mare cycle, and 60-70% for the whole breeding season for stallions.

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Thesis progress report for veterinary students

Name of student: Cristina Martinez
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Name and title of the supervisor: Dr. Horváth András DVM PhD
Department: Department of Obstetrics and Food Animal Medicine Clinic
Thesis title: The Advancements and Obstacles of Stallion Semen Cryopreservation

Consultation – 1st semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2022	2	22	Assigning topic for literature review	
2.	2022	4	15	Sumitting first draft submission and discussion of literature review	
3.	2023	5	8	Feedback of first draft of the literature review	
4.	2023	5	31	Further discussion and improvement poits for draft	
5.	2023	6	30	Submission and discussion of second draft	

Grade achieved at the end of the first semester: 5

Consultation – 2nd semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2023	7	5	Discussion of literature review doubts	
2.	2023	9	1	Discussion of literature review doubts	
3.	2023	9	30	Final thesis writing feedback	
4.	2023	10	5	Final thesis writing feedback	
5.	2024	2	27	Final thesis writing feedback	

Grade achieved at the end of the second semester: 5

The thesis meets the requirements of the Study and Examination Rules of the University and the Guide to Thesis Writing.

I accept the thesis and found suitable to defence,

.....
signature of the supervisor

Signature of the student: .....

Signature of the secretary of the department: .....

Date of handing the thesis in: 08 / 03 / 2024