

# **XVII** Congresso Internazionale SIVE

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**ATTI**  
**PROCEEDINGS**

# Basic Principles and Techniques for Semen Freezing

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The first pregnancy in a mare inseminated with frozen semen was reported in 1957 but not until the late 1980's did frozen semen in horses become more widely used. There are numerous protocols available for successful cryopreservation of stallion sperm and these protocols vary between laboratories in a number of specific elements of the process. However, there are some basic components that nearly all freezing techniques have in common. Those common steps include; dilution of neat semen in a suitable extender, concentration of spermatozoa and removal of most seminal plasma by centrifugation, resuspension of sperm in a freezing extender containing a mixture of salts, sugars, antibiotics, protein and lipid sources from ingredients such as egg yolk and milk and cryoprotective agents such as glycerol, packaging of extended sperm in straws, freezing in nitrogen vapor and storage at -197C under liquid nitrogen. Properly stored frozen semen can remain viable for many years and stallion semen frozen for more than 20 years has been used to produce normal foals. Proper thawing of frozen semen is essential and is usually accomplished by submersion of straws in a water bath at 37C. With the increased popularity of frozen semen in horse breeding it is important for equine practitioners to familiarize themselves with the process of freezing and more importantly in the proper thawing, handling and insemination of mares with frozen-thawed semen.

In our laboratories when a stallion is presented for semen freezing we first collect several ejaculates (typically 3-5) to deplete stored sperm reserves. Sperm quality (morphology and motility) and sperm production is evaluated from each

of the ejaculates to determine when sperm output has stabilized and sperm quality is maximized. Once this is achieved the stallion is given 1 -2 days sexual rest and then a "test-freeze" procedure is performed. The test freeze involves dividing the ejaculate into several portions and processing each portion using one of several protocols. In our laboratory, this normally involves four basic protocols for the first test freeze procedure. The protocols differ in the extenders used and the cooling/freezing rate employed. Straws from each of the protocols are thawed and evaluated for post-thaw motility as described below and the extender/cooling rate combination that provides the best post-thaw quality is used for the processing of additional ejaculates. If the post-thaw quality is similar between 2 or more protocols then a second or third test-freeze may be performed to confirm a consistent protocol preference. Also, if none of the four basic protocols result in acceptable post-thaw quality we will use several additional modifications of the basic protocols in an attempt to produce commercially acceptable semen doses.

## PRIMARY DILUTION

Following collection and evaluation, the first step in any cryopreservation protocol is to dilute the raw semen with a suitable extender such as basic non fat dried skim milk glucose (Kenney's) or a chemically defined media such as INRA 96. Other more complex diluents may be used if evaluation of post-centrifugation motility reveals a decrease in semen quality or if longevity studies indicate better retention of motility in an alternate ex-

tender. Semen is typically extended at a minimum ratio of 1:1 or to a predetermined final desired concentration of 50 to 100 million sperm/ml. The extender should be at a temperature of 35-37C to prevent temperature shock of the spermatozoa at the time of dilution, however once diluted, the extended semen may be processed at a controlled room temperature of 20-22C and should not be maintained at 37C.

### **CONCENTRATION OF SPERM AND SEMINAL PLASMA REMOVAL**

Stallion semen is ejaculated at a relatively low sperm concentration as compared to bull semen. Therefore in order to adequately extend semen with a freezing extender and allow packaging in small volume straws and provide manageable insemination volumes, sperm must be concentrated prior to final extension. Additionally, it has been demonstrated that high levels of seminal plasma in stallion semen is detrimental to survival during cryopreservation. The most common method to concentrate sperm and remove seminal plasma is centrifugation. Traditionally stallion semen previously diluted with extender is placed in 50 ml conical or round bottom centrifuge tubes and the spermatozoa are concentrated by low speed centrifugation (300-400 x g for 10-15 minutes). Following removal of the majority of the supernatant, this technique yields a recovery of approximately 70-75% of the sperm. Typically, 5-10% of the seminal plasma is retained along with a soft sperm pellet. Many protocols employ a centrifugation cushion of some type which involves layering a small volume of a dense, inert iodixanol preparation below the extended semen at the bottom of the centrifuge tube prior to centrifugation. The semen can then be centrifuged for longer periods (25-30 minutes) at much higher forces (1,000 x g) resulting in a greater yield of sperm recovered in the pellet. With this technique sperm are concentrated in a thin band along the surface of the cushion layer without damage to the cells caused by tight packing at the bottom of the centrifuge tube.

Recovery rates of 90 to 95% are typical using this cushioned centrifugation technique. Due to the beneficial effects of seminal plasma on mediating the immune response to sperm in the mare's reproductive tract it is critical that when concentrating sperm for cryopreservation some of the seminal plasma (5-10% is believed to be adequate) is retained in the extended semen. In an attempt to collect concentrated semen without centrifugation, open-ended AV's were developed in Poland to allow collection of only the sperm-rich fractions of the ejaculate. With this technique highly concentrated (300-500 million/ml) sperm-rich fractions can be immediately extended with the final freezing extender without the need for centrifugation. In our experience however even semen that is ejaculated at these high concentrations survives cryopreservation better when the semen is centrifuged to remove seminal plasma. One other technique to concentrate sperm involves filtration of semen through fine pore filters that allow extender and seminal plasma to pass through while trapping spermatozoa. Filtration can be stopped before complete to allow for retention of some seminal plasma. The sperm are then rinsed off of the filter with freezing extender prior to final dilution. This technique may be a simple alternative for stallions whose sperm are susceptible to damage from centrifugation. Secondary Extension with Freezing Extender Once the sperm have been concentrated and seminal plasma removed the concentrated sperm suspension is further extended to the desired final concentration with a suitable cryopreservation media. Numerous formulations exist for successful cryopreservation of stallion semen and it is our opinion that sperm from individual stallions may consistently produce preferentially better results with one extender over another. In order to produce the highest quality frozen semen from the greatest percentage of stallions in the population it is critical that technicians in a freezing laboratory have numerous validated extender formulations available and that split-ejaculate test freeze procedures are performed on all new stallions. Despite the variety of extenders used to freeze stallion semen, there are several basic compo-

nents common to nearly all of them.

1. Sugars, such as glucose, sucrose, mannose and lactose, alone or in combination provide energy for the sperm, act as non-penetrating cryoprotective agents (CPA's) and are major solutes to provide osmotic balance to the solution.
2. Proteins, typically from milk and egg yolk provide protection from "cold shock" although the mechanism of action is unclear.
3. Lipids and lipoproteins primarily from egg yolk (2-20%) closely associate with the plasma membrane surface and may act to protect sperm from freeze-thaw damage as a result of membrane phase separation and protein migration within sperm membranes.
4. Cryoprotective agents (CPA's) such as glycerol (2-5%) are critical to sperm survival. While glycerol is the most commonly used CPA for stallion sperm, others including DMSO, ethylene glycol, propylene glycol and amides such as methyl formamide have also been employed either alone or in combination to successfully freeze sperm from individual stallions.
5. Various electrolytes, buffers, antioxidants and antibiotics are also commonly found in freezing extender formulations.

The amounts of these basic components included in the various formulations varies tremendously and the optimum level of one ingredient may be dependent upon the level of another ingredient therefore a thorough understanding of the cryopreservation process and the response of sperm to freezing and thawing is essential in the development or modification of extenders. It is also important to remember that motility does not necessarily equal fertility and any new extender formulation that results in acceptable post-thaw motility must be demonstrated to also result in good post-thaw fertility before adopting that formulation in commercial freezing protocols.

## **PACKAGING EXTENDED SEMEN**

Prior to packaging the extended semen it is absolutely critical to confirm the concentration of sperm in the extended semen. Simply assuming that a certain percentage of the original

sperm were recovered following centrifugation will lead to errors in the final extended concentration and therefore the total number of sperm included in the insemination dose. This is a very common problem seen with some commercially produced frozen semen doses. Since the semen is now extended in an extender that is not optically clear a standard photometer cannot be used to determine sperm concentration. One must use a direct counting method such as a hemacytometer. This method works well as long as the pipeting and counting technique is correct and sufficient chambers per sample are counted to minimize variation and produce a reliable measurement. Data from our laboratory suggests that a minimum of four chambers must be counted to reduce the coefficient of variation to acceptable levels. This can be very time consuming so we have adopted the use of a relatively new automated counting device (NucleoCounter SP-100) that utilizes fluorescence and a DNA specific stain to rapidly and accurately count sperm in extended semen. With this technique, a small aliquot of sperm from the extended sample are diluted in a detergent solution that permeabilizes sperm membranes exposing the DNA in the nucleus to a fluorescent dye. The sperm in the sample are then loaded into a cassette that contains the DNA specific fluorescent stain causing the sperm nuclei to fluoresce in UV light. This allows for accurate counting by the device of only the sperm and prevents errors in counting due to extender components or debris. Once an accurate count is made the volume of the extended semen can be adjusted with additional freezing extender to provide the desired final concentration of sperm to be loaded into the straws.

Stallion semen has been packaged in a variety of containers including; plastic straws ranging in size from 0.25 to 5 ml, aluminum tubes, plastic packets, glass vials and pellets created from dropping extended semen onto holes drilled in blocks of dry ice. By far, the most commonly used package over the last several years has been the 0.5 ml plastic straw. These straws are closed at one end with a plug consisting of a small layer of PVC powder with cotton on either side. When the extended semen is drawn

into the open end of the straw using a vacuum it loads until the liquid passes through the first layer of cotton, wetting the PVC powder as it passes through causing it to solidify and thereby seal that end of the straw. The other end of the straw may be sealed by heat, with a plug of PVC powder or a stainless steel sealing ball. Whatever the method it is essential that an air space is left within the sealed straw to allow for the expansion of the semen as it freezes. If no space is allowed, the straw may crack or expel the sealing plug upon thawing.

### **COOLING AND FREEZING**

Once the straws are loaded they must be cooled and frozen. This is most commonly accomplished using liquid nitrogen vapor in either a passive or actively controlled freezing machine. Passive cooling involves racking the straws horizontally within the nitrogen vapor some distance above a bath of liquid nitrogen. By controlling the distance above the liquid one can control the start temperature of the cooling curve but the shape of the curve and therefore the cooling rate then proceeds at a steadily decreasing rate until the straws reach the temperature of the nitrogen vapor. Controlled rate cell freezers can be used to actively control the cooling rate and most freezing protocols involve a multi-step cooling curve to allow for cooling at different rates during different phases of the cooling curve to optimize cell survival. These systems also provide for a much more uniform and reliably consistent freezing curve from one sample to the next. One very critical component of the cooling/freezing curve is the temperature at which free water in suspension freezes (-5 to -10C) and releases heat from the exothermic reaction. The heat released can cause melting of the small ice crystals with the sperm and recrystallization of that ice into larger potentially physically damaging ice crystals. Most controlled rate freezing protocols involve a rapid rate of cooling through this temperature range in an attempt to minimize this damage. Once the straws reach a temperature of approximately -120C, they are plunged into liquid nitrogen (-196C), packaged into plastic goblets

and placed into storage immersed in liquid nitrogen until use.

### **POST-THAW EVALUATION AND QUALITY STANDARDS**

From each ejaculate frozen, representative straws should be thawed and evaluated. Most semen frozen in 0.5 ml straws is thawed in a 37C water bath for 20-30 seconds. Some thawing protocols for 0.5 ml straws involve very rapid thawing at 75C for 7 seconds. This technique works well but is impractical in field use as maintaining the water bath at 75C is difficult and inaccuracies in either the water bath temperature or the thawing time can lead to thermal damage to the sperm if exposed to elevated temperatures for even 1-2 seconds too long. It is always safe to thaw semen at 37C because sperm will not be damaged if the straws are left in the water bath too long. When receiving semen in the field practitioners should always follow the instructions provided by the semen processor for thawing. For post-thaw quality control in our laboratories, two straws are thawed for each ejaculate frozen. The contents of the straws are combined to minimize the effect of straw to straw variation and the extended semen is diluted into extender to a concentration of 20-30 million/ml. The extended semen is then incubated at 37C for 30 minutes prior to evaluating motility using computer assisted sperm motility analysis (CASA). The 30 minute incubation provides a thermal stress test that helps identify sperm that have suffered latent damage that may not be evident immediately after thawing. In addition to evaluating post-thaw motility, each ejaculate is cultured to confirm that it is free of mare pathogens or contaminating bacteria and the sperm concentration is verified using the NucleoCounter SP-100. Select Breeders Service recommends that frozen semen intended for commercial distribution should contain a minimum of 200 million progressively motile sperm after thawing with at least 30% of the sperm in the sample being progressively motile 30 minutes after thawing and incubation at 37C.