Collection, management and distribution of frozen equine semen
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Introduction
Over the last 30 years the use of frozen semen in the equine industry has increased significantly and has become a relatively common method for producing horses of numerous breeds. The increased use of frozen semen can be attributed to improvements in techniques for semen cryopreservation, availability of reliable ovulation induction drugs, increased education of practitioners on the use of frozen semen, simplified mare management techniques and major registry acceptance of foals conceived by frozen semen. Additionally, adherence to quality standards for commercial distribution has led to more positive outcomes for mare owners and veterinarians which in turn has resulted in a greater acceptance of the technique as a viable method of breeding.

Practices that wish to provide a high quality, comprehensive frozen semen service for their clients should be experienced with methods to optimize the quality of collected semen, maximize retention of semen quality through the cryopreservation process and during storage and distribution. This review will reflect on our experience and recommendations in this field.

Optimizing quality of collected semen
While it may seem to be a very elementary point, the first and most critical step in obtaining good quality frozen-thawed semen is to start with a good quality fresh semen sample. However, in my experience even experienced clinicians often have poor results freezing semen due to starting with samples that are adversely affected by improper stallion management, poor semen collection or semen handling techniques and poor hygiene protocols. Since the spermatozoa will be subjected to significant stresses during cryopreservation and thawing, any sub-lethal latent damage to the cells caused by improper handling will likely result in an inability of those cells to withstand these stresses.

Stallions that are sexually rested tend to store aging spermatozoa in their extragonadal sperm reserves and these aged sperm have reduced quality. Therefore, when collecting ejaculates for cryopreservation one should first deplete stored sperm reserves through a series of “clean-out” collections followed by one or two days’ sexual rest before collecting for cryopreservation. The number of ejaculates required to fully deplete sperm reserves varies among stallions (generally two to five is sufficient) and we recommend three successive daily collections prior to attempting to freeze. The semen quality and sperm output should be monitored and typically total sperm number per ejaculate will decrease and semen quality (both motility and morphology) will increase and become stable as the stallion’s aged sperm are depleted. Once this is done we find every other day or three times per week schedule to be best for ongoing collections of semen for cryopreservation. Although this is a general rule of thumb some stallions will tend to rapidly and significantly accumulate sperm in the reproductive tract and may require many more and very frequent depletion collections to provide the best quality spermatozoa. It is also my experience that some stallions will produce the highest post-thaw quality when collected on a daily schedule despite no observable difference in fresh semen quality when compared to less frequent collections.

Because seminal plasma makes a poor culture medium for sperm, the ideal type of ejaculate for semen preservation, either cooled or frozen, is one which has a low volume and high sperm concentration. While total sperm output is relatively constant for stallions on a regular collection schedule, seminal volume and therefore sperm concentration in the semen is greatly affected by the amount of fluid contributions from the accessory sex glands. The amount of accessory sex gland fluid in the ejaculate is influenced by the degree of sexual stimulation prior to ejaculation. Ideally, the pre-collection stimulation should be just enough to cause the stallion to ejaculate on a single mount but not so much as to cause an excessive amount of accessory sex gland fluid. Efforts should be made to deflect the stallion’s penis away from the artificial vagina to allow for the voiding of pre-ejaculatory secretions which for some stallions can contribute significant sperm-free fluid to the ejaculate volume. In a large retrospective study
of pre-freeze and post-thaw semen quality data from semen frozen by SBS laboratories Kalmar et al reported significant correlations between the number of mounts required for ejaculation and seminal characteristics. As the number of mounts required for ejaculation increased, seminal gel-free volume increased and both sperm concentration and initial total and progressive motility decreased. More importantly, as initial sperm concentration decreased, post-thaw total and progressive motility decreased (p<0.001). Multiple mounts also lead to greater risk of sample contamination with pre-ejaculatory fluids and lubricant as well as debris and bacteria from the external genitalia and abdomen of the stallion. Significant loads of contaminating bacteria will adversely affect initial and post-thaw semen quality and so proper hygiene during collection and when handling semen is critical. In a study on the prevalence and type of bacteria in extended chilled semen (Althouse), 66% of commercially prepared semen samples received by a group of clinics in the US were found to contain significant bacterial contaminants despite the antibiotics included in the commercial extenders. This illustrates the apparent lack of proper hygiene and sanitary protocols and antibiotic selection in the horse industry.

Maximizing post-thaw semen quality

Maximizing post-thaw semen quality from each individual stallion is critical to obtain consistently acceptable fertility with frozen semen. Damage from freezing and thawing can be attributed to destabilization of sperm membranes as cells move to and from storage temperatures (thermal stress) and osmotic stresses created during freezing and thawing. When external pure water in the extender freezes, the result is an increased concentration of solutes outside the cell creating an osmotic gradient between the inside and outside of the cell. Unfrozen water within the cell then moves across the cell membrane out of the cell to balance the osmotic pressure resulting in a decrease in cell volume and dehydration of the cell. This dehydration exposes the cell to high concentrations of solutes within the cell which can damage cell membranes. Additionally, as temperature drops, the lipids within the cell membranes undergo a phase transition from a more fluid liquid state to a more rigid gel state. Membrane proteins and phospholipids can reorganize in a way which leads to alterations in membrane permeability to water and ions, premature capacitation and eventual cell death.

Penetrating cryoprotective agents (CPA’s) such as glycerol, DMSO, ethylene glycol and amides are added to semen freezing extenders to minimize the damaging effects of high solute concentrations and intracellular ice formation. However, addition and removal of these penetrating CPA’s from the cells leads to large changes in cell volume as water moves into or out of the cells to balance the gradient in CPA across the cell membrane. The most common CPA in equine semen extenders is glycerol which has a permeability across the plasma membrane much lower than water, therefore glycerol moves into the cell after dilution in freezing extender at a much slower rate than water moves out leading to further dehydration of the cell during cooling/freezing. Conversely, when frozen sperm are thawed and placed in an environment free of glycerol (non-glycerol containing diluent for evaluation or mare uterine fluids after insemination), water in the environment moves into the cell much faster than glycerol diffuses out leading to rapid cell volume increases and disruption of cell membranes.

Lipid and protein sources such as egg yolk and milk are also common ingredients of semen extenders for their membrane stabilizing and antioxidant properties. Another major component of extenders are sugars such as glucose, lactose, raffinose, etc. which provide an energy source as well as act as non-penetrating CPA’s.

Species differ in the susceptibility of their sperm to damage due to cold shock and cryopreservation. These differences are thought to be related to the biochemical structure of the plasma membranes, specifically the cholesterol:phospholipid ratios, fatty acid content and membrane fluidity. It is believed that these differences are likely responsible for the variations in osmotic stress tolerance seen between species whose sperm survive cryopreservation well versus those that do not.

In addition to this species-specific variability, a well-documented inherent variation exists between individual males of many species in the ability of their sperm to withstand the stresses associated with freezing and thawing (cryotolerance). This male to male variation is especially evident in stallions. In dairy cattle, bulls have been selected by the AI industry for more than 50 years based on the ability of
their sperm to withstand the stresses of standard cryopreservation protocols. This selection has led to an increasingly uniform and positive response to cryopreservation. Studies on membrane fluidity and osmotic stress tolerance have demonstrated that bull sperm have a much greater tolerance for exposure to hypertonic conditions than stallion sperm and that there was a three-fold greater variance in osmotic stress tolerance between individual stallions than between individual bulls. Studies with boar sperm and human sperm have also revealed significant male to male variation in plasma membrane composition and some correlations have been found between cholesterol to phospholipid ratios, membrane fluidity, fatty acid content and response to cryopreservation. Further evidence for the relationship between membrane composition and cryosurvival comes from experiments with four different strains of mouse sperm that vary significantly in their cholesterol:phospholipid ratio. The percentage of motile sperm after thawing was directly correlated with the cholesterol:phospholipid ratio. The researchers were also able to dramatically improve cryosurvival in the low cholesterol strain by increasing the cholesterol content of the sperm membranes with cholesterol loaded cyclodextrins.

To date there is no single universal cryopreservation protocol that is optimum for semen from all stallions and use of a single protocol (extender, cooling rate, etc.) However, it is erroneous to group stallions into “good” and “bad” freezers based on post-thaw evaluation of semen frozen using a single common protocol. Our belief is that semen from a large percentage of stallions in the population can be frozen successfully if an effort is made to customize cryopreservation protocols to identify optimum conditions for each individual stallion. This has led to the practice of performing split-ejaculate test freeze procedures utilizing different extenders and cooling rates when evaluating a new stallion presented for freezing. It is important that the different protocols are tested on split ejaculates rather than comparing the results from one technique one day and another technique the next.

Our goals are to:

1) Produce the highest quality frozen semen from every individual stallion, not just accepting what appears to be adequate based on results from a single standard protocol and
2) Identify conditions for genetically desirable individual stallions deemed to be “poor freezers” that allows them to be included in commercial frozen semen breeding programs.

Our approach is to employ multiple protocols that are designed to determine the optimum procedure for maximum retention of semen quality after thawing of frozen semen from each individual stallion. The various extenders we use employ different sources and amounts of lipids, proteins, sugars and various penetrating and non-penetrating cryoprotectants designed to control damaging cell volume excursions during freezing and thawing.

Recently, we conducted an extensive retrospective study of data collected from stallions presented to two SBS laboratories in the United States that underwent split-ejaculate test freeze procedures during the years 1997 to 2016. The data included 1578 test freeze procedures on 1210 individual stallions of a wide variety of breeds and ages. Collected ejaculates were split into two to four fractions and frozen in 0.5 ml straws per the standard cryopreservation protocols for each individual treatment using a controlled rate cell freezer. Pre-freeze and post-thaw motility was evaluated using computer assisted semen analysis (CASA). Test straws from each treatment were thawed, diluted to approximately 25 million/ml in an appropriate extender and incubated at 37°C for 30 minutes prior to CASA analysis. Progressive motility was defined as the percentage of sperm that exhibited an average path velocity (VAP) > 50 mic/second and a straightness ratio (STR) > 75%. An ejaculate was considered “acceptable” for commercial distribution if post-thaw motility was ≥ 30%.

Overall, 81% of ejaculates subjected to the test freeze procedure resulted in acceptable post-thaw motility of 30% or greater in one or more of the extender treatments tested. If only one of the most common protocols had been used for these ejaculates only 64% of the freezes would have resulted in acceptable post-thaw progressive motility ≥ 30%. Therefore, an additional 17% of stallions were frozen successfully when the split-ejaculate test freeze method was used to select an optimum protocol.
Additionally, there was an average increase of 10 percentage points in the post-thaw progressive motility when the optimum protocol was selected versus the single standard protocol.

Post-thaw quality assessment and standards

When producing doses of frozen semen, the processing laboratory has an obligation to both the stallion owner and their mare owning clients to provide an accurate and objective representation of the quantity and quality of semen in the doses produced. While there is no officially recognized quality standard for equine frozen semen, the generally accepted industry standard is that an insemination dose of frozen semen should contain a minimum of 200 million progressively motile sperm with ≥ 30% progressive motility. We also recommend that the semen be free of known mare pathogens or a heavy load of contaminating bacteria. Since so much equine frozen semen is now being sold by the dose with little or no guarantees of fertility or quality it is important that mare owners can be confident they are purchasing doses that, at a minimum adhere to some quality standard as assessed by objective methods. This information should be available to mare owners and veterinarians so that owners may make informed purchasing decisions and veterinarians can make recommendations and management decisions to maximize the chances of success.

Sperm numbers per insemination dose must be determined accurately which requires a measurement of sperm concentration in the extended semen following centrifugation and resuspension with the freezing extender prior to packaging in straws. Because the semen is now extended in freezing extender, a standard photometer cannot be used and so a direct counting method such as hemacytometer (minimum of four chambers), Nucleocounter SP100 or CASA should be employed. Post-thaw sperm motility when determined subjectively is highly variable between laboratories and among technicians and is very subject to bias by the observer. Ideally, an objective method of determining post-thaw motility such as CASA is employed to minimize technician bias. The conditions under which the samples are evaluated is also an important consideration as variations in conditions will affect the measurement of motility. Variables that can affect measurement of post-thaw sperm motility (both subjective and objective) include; sperm concentration, diluent type, extender clarity, incubation time and temperature, stage temperature, quality of optics, chamber or slide type and technician experience. If an objective CASA system is used, the analysis parameters can be set by the user and so framing rate, number of frames analyzed and numerous other variables in the algorithms may vary between manufacturers and between two labs using the same system with different settings. Additionally, the velocity and straightness thresholds which determine if a motile cell is progressive or not may be defined by the user and differ between two laboratories. Therefore, when reporting motility information, the method of analysis and analysis parameters should be stated.

Semen storage

One of the main advantages of frozen semen is that when properly maintained at liquid nitrogen temperature (-197°C), spermatozoa can remain viable for an indefinite period, provided the spermatozoa respond favorably to the freezing process. Bovine semen stored frozen for more than 40 years has been reported to achieve pregnancies and personnel in our laboratory have used equine semen frozen for more than 30 years with good results. To maintain maximum fertilizing potential, spermatozoa must not be exposed to fluctuations in temperature and must be maintained below the critical temperature (approximately -130°C) at which thermally driven chemical reactions proceed. Exposure of cells to temperatures above -80°C can initiate some degree of thawing and then re-crystallization of ice, as well as reorganization of membrane proteins and lipids. This can lead to cell damage and impaired fertilizing potential. To maintain cells at these ultra-low temperatures, packaged frozen semen is typically immersed in liquid nitrogen. Specialized, insulated cryogenic containers have been developed that allow for such storage.

The most common type of cryogenic containers used for semen storage in the equine frozen semen industry are the smaller (20-50 liter), insulated, double walled, aluminum containers. The containers function by providing a chamber into which liquid nitrogen is loaded and the packaged semen
is immersed. Containers are manufactured by several companies and can be purchased in a variety of sizes to accommodate specific requirements. In liquid form nitrogen is extremely cold (-197°C/-320°F) and, when left in an open container, evaporates quickly into nitrogen gas. Cryogenic containers are designed to minimize the rate at which liquid nitrogen is converted to gas and allow for ultra-low refrigeration of the samples without electrical requirements. The inner chamber is constructed of aluminum which is then wrapped with an insulating foil. A second aluminum chamber surrounds the inner chamber and foil and the two chambers are welded together at the neck of the container and vacuum sealed. This construction allows temperatures within all parts of the container to remain lower than -180°C by circulating nitrogen gas within the chamber even when the level of liquid drops to a few centimeters. These tanks have round canisters with handles that hang down into the chamber from the neck of the container.

The following are recommended guidelines for the safe storage of frozen semen in liquid nitrogen containers.

1. Aluminum nitrogen containers should not be stored directly on concrete floors as this will erode the aluminum.
2. Place storage containers in a well-ventilated space, because escaping nitrogen gas will displace oxygen in a confined space and breathing air that is less than 18% oxygen could lead to a life-threatening situation.
3. Place storage containers in a room that allows for frequent visual inspection. A container that loses vacuum suddenly may exhibit a frosting around the neck of the container. If observed quickly enough it may be possible to move the semen to another container before all the nitrogen is lost.
4. Protect containers from impact as sudden impact could break the seal between the two chambers of the container and lead to a loss of vacuum and rapid evaporation of nitrogen within the container.
5. Check liquid nitrogen levels with a ruled stick at regular intervals and record the level. In our laboratory, nitrogen levels are checked weekly and nitrogen consumption recorded. A container that is starting to lose vacuum will be less efficient and increase the rate of nitrogen consumption. Detection prior to complete vacuum failure allows for the semen to be safely moved to another container.
6. Use caution when topping off containers with liquid nitrogen because the plastic straws at this temperature are extremely brittle and can easily crack causing damage that may not be revealed until the straw is thawed.
7. Prevent liquid nitrogen or objects cooled by liquid nitrogen from contacting bare skin as it can cause severe frostbite almost instantly.
8. Wear gloves and protective eyewear when handling liquid nitrogen.
9. Always dispose of liquid nitrogen outdoors by slowly pouring onto the ground and allowing the nitrogen to vaporize into the atmosphere.
10. Do not seal containers tightly as pressure build up from the expanding gas may lead to an explosion if not allowed to vent.
11. Always use a solid wooden, metal, or plastic dipstick to measure liquid levels. Never use a hollow rod or tube as the gasification and expansion of the rapidly cooling liquid inside the tube will force liquid to spurt from the top of the tube.

Accurate inventory management is critical for any storage facility or veterinary clinic holding frozen semen for clients. Small inventories can be easily tracked using a manual paper, index card or spreadsheet based system. For larger storage facilities that have a lot of inventory being added and removed for distribution, a computerized database system is useful. The key to any system is a strict policy of recording transactions that accounts for each individual straw of semen in storage. This requires that all straws entered into storage are counted accurately and that all data pertaining to those straws be entered as well. Straws should be labeled permanently with the stallion’s name, registration number,
freezing facility code or name and date of freezing or coded lot number. It is highly recommended that pre-printed straws be used rather than hand labeling as the handwriting on straws is often illegible and can lead to confusion concerning the identity of the semen. All this information as well as the semen owner, sperm concentration in the straws, number of recommended straws per insemination dose, post-thaw quality and exact location (container, canister number and section within the canister) for each individual lot should be included with the inventory.

Frozen semen distribution

Frozen semen is typically transported in a nitrogen vapor-phase container (dry shipper). These cryogenic containers maintain near liquid nitrogen temperatures (typically -180 to – 195°C) for days or weeks without the use of hazardous liquid nitrogen. Vapor phase containers work by absorbing liquid nitrogen into a thick layer of hydrophobic absorbent material that surrounds the inner cavity of the container where the semen is stored. A long holding time is achieved through the superior insulation afforded by the double-walled aluminum shell that is filled with insulating foil and vacuum sealed. Vapor phase containers must be properly “charged” by filling with liquid nitrogen to the point of saturation of the absorbent material and then pouring off the excess liquid nitrogen. Such vapor-phase shippers can be transported with a “non-hazardous” classification throughout the world, which significantly reduces transport costs. A word of caution is offered, however, concerning the use of vapor shippers; since the package is being shipped with a non-hazardous classification, it is critical that all the liquid nitrogen is poured out of the container prior to transport. Transport of a hazardous material without the proper declarations and appropriate labeling can lead to significant fines as this is a violation of IATA (International Air Transport Association) regulations and may be in breach of certain state and federal laws. Vapor shippers can be used to store semen for longer than the typical recommended holding time if more liquid nitrogen is added. If a practitioner receives semen in a vapor shipper in anticipation of inseminating a mare and she does not develop a pre-ovulatory follicle as anticipated or she ovulates before the semen arrives, the vapor container may be used to store the semen for an extended time provided the vapor container is filled again with liquid nitrogen. If this occurs, the practitioner should contact the semen supplier as soon as possible to discuss the shipper’s policy regarding tank returns.

Vapor shippers are fairly durable however it is highly recommended that they be placed inside a solid outer shipping carton that will protect the container against physical damage or loss of vacuum due to rough handling during shipping. A commonly used shipping carton has a round top and is base wide to help keep the shipper upright and prevent the package from being transported upside down or on its side. Even though it is a vapor shipper, if left on its side or upside down, the nitrogen gas will “pour” out of the container and significantly reduce holding time. Eventually, vapor shippers will lose vacuum resulting in a loss of holding time. Prior to the beginning of each breeding season it is highly recommended that all shippers be properly tested to determine if they are holding temperature per specifications. Vapor shippers and liquid storage containers that have lost all or partial vacuum can often be repaired by the manufacturer.

When packaging frozen semen for shipment, extreme care should be taken to avoid exposing straws to elevated temperatures. Use of an accurate and convenient inventory system allows the technician to quickly locate the correct straws to be loaded without having to search through canisters, trying to identify the appropriate straws. If large 5- or 4-ml straws are to be shipped they are simply loaded into the canister of a fully charged vapor shipper, taking care to always work within the neck of the storage container below the frost line as previously mentioned. Lower the frozen straws gently into the canister to prevent cracking and pack the empty space around the straws with cotton to keep them from moving inside the canister during shipment. For semen packaged in 0.5-ml straws there are two options. Straws can be loaded into small goblets and placed on canes or they can be loaded into larger plastic goblets and shipped in bulk. If straws are stored in bulk, then shipping on canes requires that the individual straws are loaded into the smaller goblets and clipped into the canes while working under liquid nitrogen. This can be accomplished with a small thick walled poly foam box filled with liquid nitrogen, under which the straws, goblets and canes are handled. While under liquid nitrogen the
technician can easily verify the identity of the semen by reading the printed straws. Printed straws held in nitrogen vapor often become frosted making them difficult to read. Straws and goblets should be handled with pre-cooled tweezers or hemostats. Likewise, when shipping in bulk, all transfers from the storage goblet to the shipping goblet should take place under liquid nitrogen. Lifting individual straws into room temperature air to transfer from liquid storage to vapor shipper should be avoided. Straws packaged in large goblets for bulk shipment should also have cotton packed around them to prevent cracking.

To facilitate proper use of the frozen semen and provide the veterinarian with information needed to manage insemination of the mare one should include specific instructions on thawing and handling the frozen semen as well as a transaction report or shipment form with the shipment. The transaction report should include the stallion’s name, number of doses, number of straws per AI dose, collection date or lot number, number of total and progressively motile sperm expected upon thawing, semen EVA status, mare’s name, breed and registration number if available and the mare owner’s information. Additional information that may be required or useful includes; instructions for return of the container, policy regarding unused doses and applicable breed registry insemination or distribution certificates.

Selected references