How to Process High- and Low-Quality Semen for Cooling and Shipment

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1. Introduction
The use of cooled-shipped semen for breeding is widespread in the equine industry, and the proper preparation of the semen is imperative to maximize pregnancy rates. Whereas some stallions have very high-quality semen that withstands cooling for transport well, other stallions require specialized processing techniques to optimize semen quality. General guidelines have been well established for processing equine semen for cooled transport, which recommends extending the semen in an appropriate extender at an extender to semen ratio of 4:1 (a minimum of 3:1 is reported in some literature) or greater to provide 1 billion progressively motile cells at a final concentration of 25 to 50 million sperm/mL. Although these guidelines are adequate for ejaculates with concentrations over 100 million sperm/mL and for sperm that tolerate cooling well, adjustments to the preparation techniques may need to be made for low-quality semen to maximize sperm quality after cooling. Ejaculates of low concentration, semen that is adversely affected by the seminal plasma, and semen that does not withstand cooling well require specialized processing techniques to optimize the quality of the sperm that is received after shipping.

Over the past several years, new techniques have been developed to help improve the quality of semen that is prepared for cooled transport. Research has shown that the use of a centrifugation cushion can improve recovery rates of sperm while maintaining semen quality and minimizing the detrimental effects that centrifugation may have on sperm. Additionally, the development of different types of extenders and the ability to cool and ship semen at 5 and 15°C provide numerous options for maximizing semen quality once it is cooled.

The purpose of this report is to describe processing techniques for the cooling and shipment of high- and low-quality semen.

2. Materials and Methods
For the purpose of simplicity, the description of processing semen will be divided into 2 parts: a review of how to process high-quality semen and an explanation of processing methods that can be used to help improve the quality of less-than-optimal semen when cooled and shipped.

3. Routine Processing of High-Quality Semen
After semen is collected, it should be handled in such a manner as to prevent shock secondary to rapid changes in temperature or osmolality. The filter
must be removed quickly from the sample to prevent contamination of the semen with the gel fraction of the ejaculate. The sample should then be extended 1:1 with an appropriate extender while calculations and measurements are performed before the final sample is prepared and packaged. Exposure to light can be detrimental to sperm; therefore the extended sample should be placed in a dark cabinet or covered. A small aliquot of raw semen is obtained from the sample to determine concentration either by a hemocytometer or an automated densimeter. If a hemocytometer is used, a 1:100 dilution of semen in buffered formal saline should be prepared for analysis (i.e., 0.1 mL of semen added to 9.9 mL of buffered formal saline). The sample is then loaded onto the hemocytometer, and all sperm within the central square that contains 25 smaller squares are counted. Both central squares should be counted. The number of sperm in the 2 chambers is divided by 2 and multiplied by $10^6$ to obtain the concentration of the sperm in the ejaculate.

There are also automated devices designed to calculate sperm concentration. Densimeters calculate concentration by measuring the amount of light that passes through a sample of semen. The higher the concentration of spermatozoa in the ejaculate, the less light that is able to pass through. A newer device available for determining sperm concentration counts the actual number of sperm cells present in a sample. The sample is mixed with a reagent that renders the sperm cell membranes permeable to propidium iodidine (PI), which is used to stain the cellular DNA. A green light is used to excite the PI and a CCD camera registers the red light emitted for correlation to a cell count. Whereas the densimeters base the concentration on density of the sample, which may be affected by debris other than sperm cells, this device only counts cells by detecting the PI-stained DNA.

Total and progressive motility of raw and extended semen are measured by placing approximately 1 mL of raw and extended semen on a warmed glass slide and evaluating sperm motility in a minimum of 5 fields. Both total and progressive motility should be determined. Motility can also be measured by a commercial motility analyzer. Total motility is defined as sperm that are moving, whereas progressive motility is defined as those moving in a fairly straight line.

Sperm concentration per milliliter and progressive motility are used to calculate a dose of 1 billion progressively motile sperm for shipment. The following guidelines can be used to easily calculate the correct volume of semen that should be extended and shipped to ensure that 1 billion progressively motile sperm are contained in each dose.

To ensure that the semen is extended at least 4:1 (some sources say at least 3:1) and that the final concentration is between 25 and 50 million sperm/mL, the following calculations are made:

- If the ejaculate has a concentration >250 million/mL, divide the concentration by 50 million/mL (the maximum final concentration that is optimal for sperm integrity) to determine the ratio of semen to extender. For example, if the concentration is 250 million sperm/mL, 250/50 = 5 total parts, yielding a dilution ratio of 1 part semen to 4 parts extender.
- If the ejaculate has a concentration between 125 and 250 million/mL, divide the concentration by 5 (the minimum number of parts acceptable in the dilution ratio). If the concentration is between 100 and 125 million/mL, divide by 4, for a minimum dilution ratio of 3:1. For example, if the concentration of the ejaculate is 195 million sperm/mL, 195/5 = 35 million sperm/mL, the final concentration of the sample when it is extended 1 part semen to 4 parts extender.

The second calculation that must be performed yields the volume of semen in milliliters that must be extended as determined from above calculations to contain 1 billion progressively motile sperm:

- The concentration of the ejaculate is multiplied by the percentage of progressively motile cells to determine the number of progressively motile sperm/mL. For example, 235 million sperm/mL × 0.65 progressively motile cells/mL = 152.75 progressively motile sperm/mL. Then divide 1000 by the number of progressively motile sperm/mL to determine the volume of semen to be extended and shipped: 1000/152.75 = 6.55 mL.

4. Example

An ejaculate containing 235 million sperm/mL and 65% progressively motile cells should be extended 4:1 with 6.55 mL of semen and 26.2 mL of appropriate extender to a final volume of 32.75 mL. This dose would contain 1 billion progressively motile cells at a concentration of 47 million sperm/mL.

Note: If the semen is extended 1:1 before analysis is performed, the same calculations as above are made, using the values obtained via raw analysis, and then 13.1 mL of extended semen (twice the amount of raw semen) should be extended up to 32.75 mL.

5. Choosing the Appropriate Extender

Another component to processing semen to yield the best-quality product after cooling and shipping is to choose an extender with which the sperm maintain good motility. Listed below are a number of different types of extenders that can be used to process equine semen for cooled-transport.

- Kenney (nonfat dried milk solid and glucose)
- Nonfat dried milk solid and glucose
6. Processing Low-Quality Semen

Because stallions are bred on the basis of their ability to perform in a certain discipline, many have less than optimal semen quality. Veterinarians must understand and be able to conduct the special procedures needed to maximize spermatozoa viability for cooled-transport. The ability to properly process low-quality semen may improve motility and longevity of the sperm after cooling.

Two problems commonly faced when processing equine semen are dilute ejaculates with concentrations of <100 million/mL and stallions whose seminal plasma is suspected of being toxic to the spermatozoa even after being diluted 4:1. If ejaculates with either of these problems are processed as previously described for high-quality ejaculates, there may be a severe negative affect on the viability of the spermatozoa.

If the concentration of the ejaculate is <100 million/mL, it is not possible to extend it 3:1 (diluting the seminal plasma to =25% of its original concentration) and maintain a final concentration of 25 million/mL, which is the lower threshold required to maintain optimal viability of the spermatozoa during cooling. It is important that the final concentration is between 25 and 50 million/mL and the final dilution is at least 3:1.

For some stallions, their seminal plasma is especially toxic to the viability of the spermatozoa even after a 4:1 dilution. The problem can be identified when semen diluted properly in numerous different types of extenders exhibits a significant decrease in viability (<20% progressively motile) after cooling during a longevity evaluation. Longevity testing can be performed by processing the semen as for cooled-transport and evaluating the motility every 6 to 8 hours for the first 24 hours, then at 48 and 72 hours. Stallions whose seminal plasma is suspected of being toxic to the spermatozoa may have a significant decrease in motility by as early as 8 hours after processing. A second method of longevity testing is performed by making 3 samples: 1 consisting of raw ejaculate, 1 extended 1:1, and 1 extended 1:4. The samples are kept at room temperature out of light and air contact. The motility is evaluated hourly until the motility is <10%.

If the motility is <10% after 3 hours, the stallion generally has poor fertility.

In both of these cases—ejaculates with low concentrations and seminal plasma that is especially toxic to the spermatozoa—centrifugation of the sample is beneficial to obtain a product that will withstand cooled transport.

Centrifugation can be performed with or without the aid of a cushion medium. The purpose of a cushion is to allow the semen to be centrifuged at a higher g-force to maximize the percentage of sperm harvested after centrifugation without having a detrimental affect on the viability of the sperm. The cushion media is a nonionic compound, iodixanol, which was first used as a density gradient or a cushion for sperm centrifugation. Whereas conventional centrifugation methods yield recovery of approximately 75% of the spermatozoa, use of a cushion can increase yields to >90%. This is especially helpful when there are low numbers of spermatozoa in the ejaculate.

If a cushion media is not available and centrifugation of a sample needs to be performed, the following protocol is used. The ejaculate should be extended 1:1 with extender, placed in 50-mL conical tubes at a volume of 40 mL/tube, and centrifuged for 10 to 12 minutes at 400 to 600g. If the centrifuge being used is calibrated in RPMs instead of g-force, the RPMs necessary to obtain a desired g-force can be calculated by measuring the radius of the rotor arm (in cm) from the center of the centrifuge to the end of the rotor bucket and applying the following formula:

\[
N \times g / 0.02236 \times R \\text{ (rotation radius in cm)}
\]

Once centrifugation is completed, remove 30 mL (75%) of supernatant from each tube and then re-extend the pellet by adding 20 mL (75% of the volume removed) of extender. This will yield a concentration similar to the original concentration.

If the original concentration was >50 million/mL, additional extender will need to be added; if it is <25 million/mL, less extender should be added. The following calculations can be used to determine what that volume should be when using 25 million/mL as the final concentration for very dilute samples and 50 million/mL for more concentrated samples.

- Glucose and sucrose
- Purified fractions of milk caseins
- Egg yolk–based
- Defined caseinates and whey proteins
- Soybean lecithin

Although this is not a complete list of all of the equine semen extenders available, it demonstrates the variety of proteins and sugars used to formulate different extenders.

Whereas some stallions’ semen does relatively well in a number of different extenders, other stallions’ semen maintains progressive motility much better in one type of extender rather than another. To determine the best extender for a stallion, a test cool should be performed, using several different types of extenders; the semen should be packaged as if for shipping and motility analysis performed on the samples 24, 48, and 72 hours after processing.

For good-quality semen, choosing a suitable extender and processing the semen properly should yield good-quality cooled-shipped semen (minimum of 1 billion progressively motile sperm per dose extended a minimum of 4:1 [semen:extender], with a final concentration of 25 to 50 million sperm/mL).
Original concentration

\[ \times \text{original volume of ejaculate} \]
\[ = \text{Total number of sperm in ejaculate} \]

Total number of sperm

\[ \times 0.75 \text{ (a recovery rate of 75\%)} \]
\[ = \text{Total number of sperm recovered} \]

Total number of sperm recovered/(50 million/mL)

\[ = \text{Final volume of extended semen} \]

The sperm recovered from each tube will be pooled and extended together to yield the above calculated final volume of semen. The extended sample should then be analyzed for motility after centrifugation to determine what volume is required for a dose of 1 billion progressively motile sperm.

For example, if the final extended concentration is 50 million/mL and the progressive motility is 65\%, there are 32.5 billion progressively motile sperm/mL: 1000/32.5 = 30 mL (30 mL of extended semen equals 1 dose of 1 billion progressively motile sperm).

To maximize the percentage of spermatozoa obtained, 1 mL of a cushion media can be layered via positive displacement in the bottom of a conical tube, using a pipette after the extended semen has been added (Fig. 1). The sample is then centrifuged for 20 minutes at 1000 g and the supernatant is removed quickly down to between the 7.5- and 5-mL mark. The cushion fluid is then removed from beneath the pellet using a 0.5-mL semen straw (Fig. 2). The sample can then be re-extended using the same calculations as above. It has been recommended that glass nipple-bottom tubes be used if the sperm numbers in the ejaculates are less than 2 to 3 \( \times 10^9 \) or when it is necessary to separate more seminal plasma from sperm than is possible with cushioned centrifugation in conical-bottom tubes. Special rotor buckets are required to hold these tubes and are typically not available in the general practice.

Once the semen is properly prepared for cooling and transport, it should be loaded into all plastic syringes or conical tubes anaerobically (without any air in the syringe or tube) and shipped in a container designed to transport cooled semen. Although both non-disposable cooled semen shippers and disposable Syrofoam shippers are adequate for transporting cooled semen, research has shown that the non-disposable shipper is able to cool the semen at a more consistent rate and hold the desired temperature for a longer period of time. When shipping semen of lower quality, it may be beneficial to use a non-disposable shipper especially when weather conditions are extreme.

A small number of stallions do not tolerate cooling of their sperm well, and a protocol has been described using an extender formulated from purified fractions of milk caseins. Although this is not a widely used technique, it is important to be aware of as another option when attempting to maximize viability of low-quality semen.

7. Results
The use of proper processing techniques to ensure that an adequate number of spermatozoa are shipped is imperative to obtaining good fertility results. Although some stallions with good fertility can achieve excellent results with much fewer progressively motile sperm than other stallions, one should not ship less than the standard 1 billion progressively motile sperm unless it has been determined that less is acceptable for a particular individual.

Using techniques such as centrifugation and products such as cushion media allows us to ship ejaculates that would not normally tolerate transport well. Performing test cools with different extenders permits us to determine the best type of extender for a stallion and thereby potentially improve the quality of the product received after cooling. Research has shown that many samples of cooled-transported semen received do not contain the
accepted number of total or progressively motile sperm. Other samples are too dilute or were not centrifuged when centrifugation was required. All of these factors can have a negative impact on pregnancy rates.

8. Discussion

When preparing semen for cooled transport, it is important to recognize that all ejaculates are not of the same quality and that all spermatozoa do not tolerate cooling. The best product can be produced when the ejaculate is packaged at the correct concentration and dilution ratio with an appropriate extender. Each stallion should be treated as an individual, and extended aliquots from every ejaculate should be analyzed at 24 and 48 hours after shipping to ensure that the spermatozoa have maintained their integrity.

Educating stallion owners about the importance of a test cool before shipping cooled semen allows them to determine the best protocol for processing that stallion’s ejaculate and what changes may need to be used to improve semen quality.

A process not discussed in this report, due to the lack of research available on its use for cooled shipped semen, is gradient centrifugation. Centrifugation of equine semen through a silica-particle solution has shown promise for selecting sperm with good motility, morphology, and chromatin quality. Currently, this procedure is used to improve fertility and pregnancy rates for stallions with a large number of morphologically abnormal sperm. Two to 4 mL of the bottom layer of the silica-particle solution is placed in the bottom of 15 mL conical-bottom tubes and approximately 1 mL of extended semen containing $500 \times 10^6$ are layered on top. The sample is then centrifuged, and the silica-particle solution and supernatant is removed from the pellet containing the high concentration of morphologically normal sperm. Due to the low number of sperm recovered, this technique is currently mainly used for low-dose insemination; however, protocols may soon be developed that allow this procedure to be incorporated into the processing of low-quality semen for cooled-transport.

References and Footnotes