"Boys in a box"- chilled and frozen canine semen shipments

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Abstract

Canine semen used for shipping or cryopreservation should meet minimal semen evaluation standards for concentration, motility and morphology to maximize artificial insemination success. Samples with increased morphological abnormalities may benefit from filtration or gradient centrifugation; however, total numbers will be much lower. Extenders are added to sperm-rich fraction to supply energy source, buffer the environment, decrease bacterial growth and protect sperm membranes during chilling and freezing. Many commercial and homemade extenders are available, along with various shipping box options, to successfully transport semen virtually anywhere in world. Additional cryoprotectants are added to frozen semen extenders to prevent ice crystal formation within sperm. Standardized protocols and routine maintenance of nitrogen tanks, and vapor shipper dewars increase chances of successfully protecting, storing and shipping canine semen.

Keywords: Chilled semen, cryopreservation, semen extenders, canine, dog

Introduction

It has become very common to use chilled and frozen semen for canine artificial insemination, due to convenience and advantages of shipping semen instead of costs and risks of shipping bitches or dogs to be bred, or driving long distances for a natural breeding. Frozen semen can store genetic material of breed lines for later use and also helps expand genetic diversity. Protocols must be followed when shipping or receiving chilled or frozen semen to ensure highest semen quality at insemination.

Stud dog

Goal of breeding is to supply healthy puppies for companionship, service, etc. Therefore, all dogs collected for chilled or frozen semen use should have a normal physical examination, be current on health clearances and vaccinations and be *Brucella canis* negative. A DNA number is required by AKC in order to register puppies born using chilled or frozen semen. This is easily obtained by mailing a cheek swab. To maximize chances for a litter, only semen that meets minimal evaluation parameters in regards to concentration, motility and morphology should be chilled or frozen. For international shipments, collecting veterinarian should check with USDA regarding stud dog mandatory health testing required PRIOR to collecting semen to meet importation requirements for destination country. Current recommendations are available online (www.aphis.usda.gov).

Semen preparation

Presence of first or third fractions of ejaculate may adversely affect semen motility and morphology.[1] These 2 fractions, along with urine, can be removed with supernatant after centrifugation. Regardless, very effort should be made to capture only second (sperm rich) fraction during collection.

Filtration procedures or centrifugation gradients may improve semen quality. With filtration, ejaculate is passed through a filter (e.g. glass wool). Using a centrifugation gradient, sample is carefully pipetted on top of a layer of specialized media in a test tube and centrifuged. Normal sperm move through media and remain on tube bottom as a pellet while media binds abnormal sperm. Some media may aid in blood removal from sample, particularly digested blood, and centrifugation media can also be used to remove urine. These 2 procedures may improve percent morphologically normal sperm; however, drastic reduction in total numbers, especially with glass wool filtration, may preclude shipping or freezing that sample.[2,3]

Extenders

Canine semen can be shipped at refrigerator temperatures (4 - 5ºC) and used within a few days or frozen and stored in liquid nitrogen (-195ºC) to be used until years later. Regardless, sperm-rich portion will need to be mixed with an extender, a liquid preparation supplying multiple benefits.

Semen extenders contain energy source (e.g. glucose or fructose[4]) to support motility and contain buffers to help stabilize pH and osmolarity of environment offsetting acid-base changes of metabolism during storage.[5] They also contain a protein source (e.g. egg yolk or milk) to protect sperm membrane integrity during temperature fluctuations during chilling, freezing and thawing. Percent progressively motile sperm declines gradually with time in extended samples chilled to 4 - 5ºC, with slower decline in extenders containing 20% egg yolk compared to skim-milk based extenders.[5]

Commercial preparations have ingredients that are often proprietary and vary with their composition (Camelot Farms, College Station, TX; International Canine Semen Bank, Sandy, OR; Synbiotics, Kansas City, MO; CLONE, Doylestown, PA; and Minitube, Verona, WI). Many formulas available for homemade extenders; however, these have increased chances for more variability between batches.

Preparing and shipping chilled semen

Prior to shipping, inseminating veterinarian should inform collecting veterinarian about type of insemination being done and preferred volume desired. Proper labeling of all plastic or glass tubes containing semen with permanent marker is paramount! The Society for Theriogenology has guidelines on their webpage (www.therio.org) regarding tube identification and paperwork recommendations to ensure correct identification. Records annotating semen evaluation results, extender used, dilution, volume, etc. should be in duplicate, with clinic retaining a copy. An established clinic protocol should be followed for every shipment.[6]

Collected ejaculate is centrifuged (300 - 700 x g for 5 - 6 minutes) if it contains any of first or third fractions, after which supernatant is withdrawn and extender added (at similar temperature as semen). Most commercial extenders recommend a dilution of 1 part semen to 4 parts extender. Usually, 2 - 6 ml for intrauterine or a transcervical insemination and 1 - 10 ml for vaginal inseminations are shipped, depending on bitch size and amount requested by inseminating veterinarian. A small aliquot should be removed and refrigerated until shipped sample is evaluated upon arrival. This saved sample can be used to evaluate semen if it arrives in poor condition at insemination facility to determine if semen did not chill well or if something happened to the box after leaving collection facility (i.e. temperature extremes). After cap tube is secured, tube top is wrapped with Parafilm® (Bemis Company, Neenah, WI). Scotch tape, strapping tape, and duct tape should not be used as they do not provide a watertight seal. Plumber's tape, if used, should not have any contact with semen as it may be spermicidal (depending on brand).

To maintain a cool temperature, most commercial semen transport boxes are lined with extruded polystyrene foam and use 1 or 2 plastic liquid bricks that will need to be frozen prior to shipping. To ensure that extended semen does not come into direct contact with frozen brick, tube and frozen brick(s) should be thoroughly wrapped with either several layers of newspaper or some form of packing material, e.g. bubble wrap. Chilled canine semen shipped in Equitainer® (Hamilton Research Inc, South Hamilton, MA), a plastic shipping container that is primarily used to ship stallion semen, had highest percent motility 48 hours post-chilling compared to a styrofoam box or a Thermos flask[7]. This container is also approved for multiple uses.A new thermos system called Ship-Mate® (My Breeder Supply, Elk City, OK), is an electronic-chilled vacuum flask that uses a battery inside a thermos to maintain temperature at 5 Cº for duration of transport and can record temperature. Research is needed to verify accuracy.

Chilled semen should be shipped as quickly as possible and maintained between 4 - 5ºC to ensure optimal quality upon delivery. Fedex and UPS are both reliable for pickup and overnight deliveries. When shipping on a Friday for a Saturday delivery, be sure to mark appropriate box on shipping label to avoid delivery on Monday. When shipping on Saturday for a Monday delivery, be sure to mark the waybill as an overnight shipment; otherwise, it will be delivered on Tuesday if marked as 2 day shipment.

Receiving a chilled semen shipment

A chilled semen shipment should be opened upon arrival and frozen bricks inspected. If they are warm, semen temperature is most likely too warm and could influence semen quality and bacterial growth. After 48 hours, extended canine semen maintained at 4 - 5ºC with antibiotics effectively controlled aerobic bacterial growth, but not when held at room temperature.[8]

A drop of extended semen should be warmed on a 37ºC slide warmer and examined for motility, morphology and a general estimate of total numbers to compare to data on shipping form. Any large discrepancies should be reported to collecting veterinarian to allow for changes in future planned shipments, e.g. using a different extender. Stud dogs that have never had semen chilled should have a "chill test" done several weeks prior to shipping where sperm rich fraction is extended using various extenders, refrigerated overnight and motility and morphology verified 24 hours later to determine the extender that maintained best quality overnight.

If insemination cannot be performed immediately, extended semen should be refrigerated and used as soon as possible. Extended chilled semen is best used within 48 hours of collection.[5] Canine semen tends to tolerate chilling very well and may often maintain excellent motility for many days (sometimes up to 8 - 10) regardless of extender used. You do not have to use a specific 5 or 10 day extender to expect similar results; most semen that ships well in a 5 or 10 day extender will ship just as well in many other extenders. Some of 5 and 10 day extenders seem to promote agglutination of sperm to coverslip upon warming; although this does not appear to affect fertility, it makes it difficult to accurately assess motility.

Frozen semen principles

Frozen semen extenders are very similar in makeup to chilled semen extenders, except most contain glycerol and egg yolk as cryoprotectants to stabilize and protect sperm exposed to the damaging temperatures of liquid nitrogen. During cryopreservation, glycerol, ethylene glycol, or dimethylsulfoxide[9] permeates through sperm plasma membrane, interacts with water molecules, and inhibits formation of hydrogen bonds necessary for formation of damaging ice crystals. Egg yolk contains lipids lecithin and cephalen[5] that are too large a molecular weight to cross cell membranes; therefore, ice crystal formation is inhibited by causing cellular dehydration, freezing point depression and increasing media viscosity at lower temperatures.[9,10]

Semen is slowly cooled to 4 - 5ºC and then frozen in either straws or pellets. Prelabeled polyvinylchloride straws, 0.25 or 0.50 milliliters in volume, are filled with cooled semen and an air bubble located centrally in straw. Straws are then sealed and frozen by suspending them over liquid nitrogen vapor and then plunging them directly into liquid nitrogen.[5] Straws are placed in canes and stored vertically in goblets in liquid nitrogen cryotanks. Pellets are made by placing individual 50 - 100 µl drops of cooled semen into small depressions made in dry ice and then immersing pellets into liquid nitrogen and storing them in pre-labeled cryovials.

Decision to freeze semen in pellets versus straws is personal choice. Pellets are convenient in that the entire vial, usually a calculated amount to breed 1 bitch, is thawed, whereas straws are thawed individually. Pellets take less room to store, as vials are packaged in a box and several boxes fit on a cane; each goblet can only hold ~ 6 - 8 canes of straws. Straws, however, are more accepted internationally. Straws also prevent cross contamination with anything that may be in the liquid nitrogen and make it impossible to combine or mix semen from 2 animals together.

Vapor shipper dewars

Frozen semen is shipped in a "dry shipper" dewar, a vacuum-insulated canister lined with a hydrophobic material that absorbs liquid nitrogen, thereby keeping the frozen semen stable in nitrogen vapor during transport. Liquid nitrogen itself is labeled as a hazardous material by US Department of Transportation and cannot be used for shipment, making it necessary to use a dry shipper to transport semen.[11] Canister is housed in an outer heavily padded, plastic container that helps keep the canister upright during transport.

A face shield and protective insulated gloves should be worn when working with liquid nitrogen. Canister will need to be filled, or "charged" 24 - 36 hours prior to a shipment, by slowly adding liquid nitrogen to canister using a funnel until level reaches bottom of the neck. Care must be taken not to spill liquid on vacuum cap as this may cause shrinkage of seal allowing air to leak into vacuum space. Neck plug and lid are replaced and canister is allowed to sit dormant so nitrogen can permeate absorbent. This process is repeated until level of liquid nitrogen no longer falls. Weigh shipper and check manufacturer's manual as there are suggested full weights to indicate normal absorbent filling[12] and holding times.

Evaporation rate of a vapor shipper is calculated to monitor charging capability. Vapor shipper is charged, liquid nitrogen dumped out and tank weight recorded. Canister is weighed again after sitting dormant for 24 hours and second weight recorded. Evaporative loss per day is calculated by multiplying difference between 2 weights by 0.5606. If this number is > 1.9 (liters/day), vapor shipper should either be replaced or sent to manufacturer to be revacuumed. Vapor shippers should also be weighed prior to shipping and when they return, to confirm that evaporative rate/day is within acceptable limits.

Frozen semen shipment

Just prior to shipping, tank needs to be inverted over an insulated bucket and held until liquid no longer runs out. Canister neck plug and lid should be replaced and canister watched for 1 hour to be certain no condensation or frost collects around top that could indicate a leaking tank. Canes loaded with straws or cryovials are then transferred to goblet inside canister, and neck plug and lid inserted. Lid is then anchored shut with a zip tie. All paperwork (copy of freeze report, instructions for thawing, and return Fedex label) is placed in a manila envelope next to canister, along with any necessary supplies for inseminating veterinarian and outer container is closed and anchored with a zip tie, or left unanchored if canister needs to be sealed by US Department of Agriculture for an international shipment. Like chilled semen, Fedex or UPS are preferred carriers and tracking numbers should be sent to receiving veterinarian.

Receiving frozen semen

When you receive a frozen semen shipment, container should first be inspected for damage. It is then opened and inner canister lid carefully removed watching for a vapor "puff," confirming that canister is still charged. Contents should be quickly transferred to a bucket of liquid nitrogen, carefully inventoried to verify shipment of correct semen and then transferred into a regular liquid nitrogen storage tank, as shipper stability is unknown. If you do not have any cryostorage tanks and need to hold frozen semen a few days before using, it is best to either add liquid nitrogen to dry shipper daily or arrange for arrival of frozen semen on insemination day. Before returning canister, remove goblet and invert it over cryobucket to ensure that a cryovial or straw did not dislodge from cane during transit.

Closely examine cryovials containing pellets to be sure that vials themselves are not fractured into tiny shards and that semen is still in a round, pelleted form and not a thawed glob on vial bottom. Straws should also be examined for damage such as cracking or extrusion of end plugs. Any signs of tank failure or damage should be immediately reported to vapor shipper facility.

Maintenance of storage canisters

Liquid nitrogen level in storage tanks should be measured weekly using a calibrated cryostick and recorded in a log to monitor evaporation loss prior to topping them off. Any tank evaporating at a faster rate than previously noted should be removed from service.

Conclusion

Canine semen is routinely being shipped everywhere in US as well as internationally, allowing ease of breeding and increasing availability of diverse genetic material. There are many extenders and shipping box options available for chilled semen to preserve semen quality during transport. Frozen semen can be stored indefinitely, allowing access to several genetic lines. A great deal of paperwork and maintenance is involved when dealing with semen shipping and freezing to ensure quality and accuracy.

Hopefully, evidence-based research will provide standardized protocols for canine semen shipping, cryopreservation and insemination in near future to increase chances of producing healthy, normal-sized litters.

Conflict of interest

There are no conflicts of interest to declare.

References

1. England CW, Allen WE: Factors affecting the viability of canine spermatozoa. II. Effects of seminal plasma and blood. Theriogenology 1992;37:373-381.
2. Robert MA, Jayaprakash G, Pawshe M, et al: Collection and evaluation of canine semen-A review. Int J Environ Sci Technol 2016;5:1586-1595.
3. Root Kustritz MV: Applied small animal andrology. In: Chenoweth PJ, Lorton SP, editors. Animal Andrology Theories and Applications. Wallingford (UK); CAB International: 2014. p 177-196.
4. Ponglowhapen S, Essen-Gustavsson B, Forsberg CL: Influence of glucose and fructose in the extender during long-term storage of chilled canine semen. Theriogenology 2004;62:1498-1517.
5. Johnston SD, Root Kustritz MVR, Olson PNS: Semen collection, evaluation and preservation. In: Canine and Feline Theriogenology. W.B. Saunders; Philadelphia: 2001. p. 298-304.
6. Escobar SR: Adding reproduction to your practice: the good, the bad and the ugly. Proc Society for Theriogenology Annual Conference and SFT/ACT Symposium 2004; p. 237-239.
7. Lopes G, Simoes A, Ferreira P, et al: Differences in preservation of canine chilled semen using different transport containers. Anim Reprod Sci 2009;112:158-163.
8. Barstow C, Root Kustritz MV: Effect of antibacterial agents in semen extender on bacterial growth in extended canine semen held at 5 degrees C and 20 degrees C for up to 48 hours. Clinical Theriogenology 2014;6:231-238.
9. Eilts BE: Theoretical aspects of canine semen cryopreservation. Theriogenology 2005;64:692-697.
10. Holt WV, Penfold LM: Fundamental and Practical Aspects of Semen Cryopreservation. In: Chenoweth PJ, Lorton SP, editors. Animal andrology theories and applications. Wallingford (UK): CAB International; 2014; p. 76-99.
11. Dry Shippers. University of Washington Environmental Health and Safety Pamphlet. 2002.
12. MVE Operating Instruction Manual for Vapor Shipper Dewars; Ref 11562640 Rev D;6/05. p. 2-9.