Technique Report

Handling techniques of frozen semen: storage, transfer, transport, and thawing*

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Abstract

Enormous demand for assisted canine reproduction services in the last decade has increased the use of frozen semen in veterinary clinics. However, training and knowledge regarding frozen semen handling, as observed by the author, has been lagging. Therefore, this report focuses on simple steps to avoid damage to frozen semen prior to its use.

Keywords: Frozen semen handling, dryshipper, semen damage, semen storage

Introduction

Damage to frozen semen prior to artificial insemination by improper storage or handling are underestimated causes of pregnancy failure. Since there is an increase in demand for frozen semen use in dogs, many general practitioners have added frozen semen usage in their practice. Rapid and invisible damage may easily occur to cryopreserved sperm if storage, handling, and shipping procedures are performed by untrained personnel.

The safest place for frozen semen is when they are immersed in liquid nitrogen at a constant temperature of –196°C. Nitrogen vapor, alone, does not protect frozen semen from the harm due to temperature variations within the vapor. Nitrogen vapor temperature varies depending on the surface area of evaporation and the distance from the liquid nitrogen surface. Damage can occur to frozen semen when moved from one tank to another due to varying temperatures of the room environment air and the nitrogen vapor in the tank.

Studies in the early 70's using bovine frozen semen demonstrated that recrystallization (a short thawing and refreezing of ice crystals that occur in a frozen semen sample when an inside-temperature of –130°C and higher is reached within the straw) can damage sperm.[1,2] Temperature of –130°C within a straw may be reached faster when semen is handled in vapor. This report focuses on describing possible errors while handling frozen semen and simple ways to prevent them in a veterinary practice setting.

Semen storage in liquid nitrogen tanks

Frozen semen is stored in liquid nitrogen at –196°C. A liquid nitrogen tank, also called a wet-tank or LN2 tank, is a double-walled container where the inner tank is suspended from the outer shell by a neck-like structure. Vacuum between these 2 tanks helps to insulate the inner tank so that nitrogen in the inner tank holding the semen evaporates much slower. Depending on its size, a tank in a veterinary clinic usually contains between 6-10 canisters that are held at the neck opening and carry canes with a lower and an upper goblet containing semen straws or vials of semen pellets. A great advantage of a liquid nitrogen tank is that it does not depend on electrical power, unlike other freezers. However, tanks need regular supervision since liquid nitrogen evaporates and the level of the liquid decreases over time. The plug is designed to loosely fit in the tank's neck to allow vapor to escape. A plug should never get wet with water; otherwise, it may freeze to the tank's neck. If capped too tightly, the gas builds pressure in the tank and causes it to crack open or even 'explode' eventually. Since there is continuous evaporation, liquid level should be measured weekly with measuring sticks especially designed for that purpose. The measuring stick should be lowered to the tank bottom, allowed to cool, and then withdrawn. The level of nitrogen is represented by the frosty layer on the measuring stick.

All measured levels and the amount of new nitrogen added should always be recorded not only for insurance purposes

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but also to assess the continued performance of the liquid nitrogen tank. A sudden increase in evaporation rate may be a sign that damage has occurred to the insulating properties of the tank. Stored semen may be lost if tanks are not supervised regularly. Semen tanks should be stored away from heavy traffic of personnel in a well-ventilated area due to the continual evaporating nitrogen gas. Tanks should be placed on a smooth surface, ideally on a dry platform with rollers, so that they can be easily moved. Liquid nitrogen tanks should always be carried by 2 people using both handles so that the tank remains in a vertical position to avoid shaking the canister rods inside that could potentially damage the fragile tank's neck. At the same time, the tank should be visible enough every day so that any sudden vacuum loss and a higher evaporation rate can be noticed. Typically, damage of the vacuum can be noticed by frost accumulation around the lid of the tank and, in severe cases, all over the area where the plug closes the tank. It is very important to maintain an accurate and updated semen inventory. Special programs for inventory are offered by various companies but simple 'Excel sheets' are sufficient and are easy to follow by personnel in private clinics. Owner, tank and canister number, cane tag color and tag labeling, reception date, and removal dates must be entered. A good inventory saves time to find a specific semen dose; most importantly, it protects the quality of sperm. It must be kept in mind that each unnecessary lifting of a canister and searching for the right cane may cause damage to all semen in the canister over time.[2] Damage to frozen semen starts at inside straw temperatures as low as –130°C.[1] Recrystallization was definitely observed by –80°C, occurring within a matter of a few hours of exposure at –57°C, within a few minutes at –48°C and within a few seconds at –30°C. Nitrogen vapor temperature can vary from –190 to ~ –30°C depending on how far from the liquid/vapor interface and how close to ambient air the measurement is taken (Table 1). It took only 10-15 seconds for the temperature of a 0.5 cc straw in a vapor filled goblet to drop to –100°C when the goblet was raised to the upper third area in tank's neck.[3] Semen exposed to reach an inside temperature of –45°C resulted in a dramatic decline in postthaw motility.[1] Invisible semen damage occurs before a drop in postthaw motility is noticeable during a routine semen evaluation, making preventive measures when handling semen so important.[4] In large animal practices, where daily inseminations and embryo transfers are common, these findings are known as the reason for the so-called '8 second rule', stating that semen should not be lifted beyond the frostline (Table 1) and be limited to no more than 5-8 seconds in the neck of a tank during a search.[4] Obviously, this rule must be applied to frozen semen of all species. A warmer temperature is reached much faster when the liquid nitrogen level is low compared to searching for semen in a fully charged nitrogen tank. It is clearly demonstrated that repeated exposure of canes to the neck is more harmful in a tank that has a low level of nitrogen compared to a full tank and if this exposure is repeated the harm is additive and irreversible (Figure 1). Again, depending on the frequency and duration of damage due to these types of mishandling, a decrease in postthaw motility cannot always be observed; however, fertility decline has been noticed when frozen semen samples had been handled incorrectly in bovine practice. Author has measured the vapor temperature at the neck of 6 semen tanks (all Model MVE XC 20) with a thermocouple device. A tank with a liquid nitrogen level of only 10 cm had a neck temperature of –54°C compared to –89°C in the same model tank with a level of 30 cm of liquid nitrogen. Again, this simply means that search of a specific semen cane is safer when the tank has a high level of nitrogen. In addition, with a level of 30 cm, the upper goblet or cryovial in an MVE-XC20 tank contains still liquid nitrogen that keeps the semen at –196°C when withdrawn or lifted for a short time. Therefore, before searching for semen, it is safer to make sure that any tank has or is being topped up to a liquid nitrogen level that covers the upper goblets with semen portions.[2]

Table 1. Example of temperature range in the neck of a 20-liter semen storage tank filled up to 75% with liquid nitrogen (*frostline of the tank); adapted from Stroud: 2013[4]

Depth in Neck	Temp °Celsius
0 inches	24.1
–1 inch	21.9
–2 inches	11.9
–3 inches	–23.0
–4 inches*	–47.1
–5 inches	–73.6
–6 inches	–110.5
–7 inches	–160.4
–8 inches	–181.2

Transfer of semen from one tank to another tank

When transferring semen from one tank to another tank, semen passes shortly through nitrogen vapor and air and then back into vapor and/or liquid nitrogen; this must be done swiftly. Again, a good inventory to locate the semen is crucial to avoid lifting canisters unnecessarily. The next important step is to have both tanks side by side to do the transfer. Shipments often contain semen from several dogs with a varying number of doses per insemination. Author usually transfers all received semen into a shallow styrofoam dish with liquid nitrogen so that the name and number of doses can be confirmed before transferring canes into the clinic's tank. This is still the safest method. If just 1 dose on a cane is received, it is possible to transfer it swiftly from one tank to the other. Although canes can be touched with a gloved hand, straws should not be handled with fingers but only with LN2 precooled forceps to avoid rising temperature within the straw.[3]

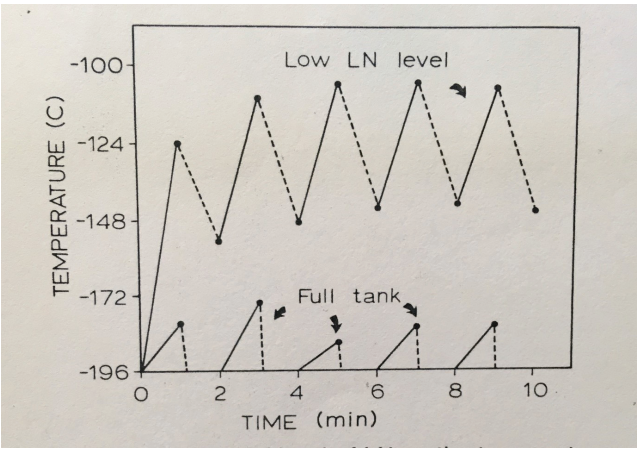

Figure 1. Influence of level of liquid nitrogen on the temperature within semen straws (0.25 cc) during repeated lifting and exposure to the neck of a liquid nitrogen tank (Picket et al., 1976[2])

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Again, the '5-8 second rule' applies for any transfer through vapor to avoid recrystallization of frozen semen.

Preparation and handling of frozen semen in a vapor shipper (commonly known as dryshipper)

Dryshippers were designed for the safe transportation of frozen semen or other biological samples at cryogenic (–150°C or colder) temperatures. They are manufactured from durable, lightweight aluminum and employ a hydrophobic and porous compound that absorbs liquid nitrogen to ensure dry, spill free vapor-phase shipments. These shippers are the ideal way to ship frozen semen safely and, since there is no liquid nitrogen inside, they are not considered 'dangerous goods' when transported on ground or by plane.

As mentioned before, nitrogen vapor is not always a safe place for frozen semen. When dealing with dryshippers, meticulous preparation is required to ensure a damage free transport of frozen semen. It is important to follow the instructions of the manufacturer, especially when filling a tank that has no vapor left. Whenever either a LN2 tank or a dryshipper is filled with liquid nitrogen, the liquid should be added slowly. Personnel should wear protective glasses or a face shield and insulated gloves since a significant amount of gas is generated and liquid may reflux out towards the person when it comes in contact with the ambient temperature in the shipper. It is advisable to fill such tanks with small amounts at a time until the dryshipper is cool enough inside. This is also important to avoid any cracks inside the tank due to sudden cooling when initially loaded. The tank can be filled to the neck once well cooled, the cap replaced, and then it should be put aside for a few hours to let the liquid be absorbed. When all liquid is absorbed, new nitrogen may be added. If nitrogen liquid is bubbling in the dryshipper, it means that nitrogen is still being absorbed into the porous membrane. Once the bubbling stops, it can be assumed that the porous membrane is saturated. The remaining liquid must be removed completely, and the dryshipper is ready to receive the frozen semen samples. Since liquid nitrogen is classified as 'dangerous goods' by the International Air Transport Association, all liquid nitrogen must be removed completely from the dryshipper so it can be identified as 'Not Restricted' as per special provision A152 on the shipping waybill. Personnel must be trained to work safely with liquid nitrogen to avoid any accidents. Liquid nitrogen that spills on the skin may cause severe burn wounds. Therefore, violation of these regulations can result in civil or criminal charges. Dryshippers are usually shipped in a protective case to avoid any damage during transport. Author uses only the round, mushroom shaped protective case because the dryshipper is transported in an upright position. There are also rectangular protective cases. However, the author has received several dryshippers in these cases that were almost out of vapor, although the shipper confirmed adequate charge before shipment. When the dryshipper is transported or stored horizontally in these rectangular cases, there is a faster evaporation rate and the holding time may be shorter compared to tanks that are transported upright (personal observation).

Again, nitrogen vapor alone does not mean that the semen is at the right temperature. In the dryshipper, there is no level of nitrogen that we can measure with a measuring stick. However, the weight of a dryshipper is correlated with the absorbed liquid nitrogen in its inner porous membrane. Manufacturers provide us with specifications for each nitrogen tank, i.e. with the specific weight when a dryshipper is fully loaded or when fully dry (Table 2).

Table 2. Specifications of various dry shippers as published online by the manufacturer of MVE models

Vapor Series	**SC 2/1V**	**SC 4/2V**	**SC 4/3V**	**SC 20/12V**	**XC 20/3V**
Static Holding Time Days	**8**	**13**	**21**	**85**	**16**
Maximum Storage Capacity					
No. of Canisters	1	1	1	6	4 +1 Center
No. of 1/2 cc Straws 10/cane	-	280	120	540	2500/2000**
No. of 1/2 cc Straws 1 Level Bulk	88	440	210	780	3750/3000**
No. of 1/4 cc Straws 1 Level Bulk	182	938	452	1630	7410/6000
No. of 1.2 & 2.0 ml Vials 5/cane	-	95	40	150	675/560**
No. of 1.2 & 2.0 ml Vials 6/cane	9	106	48	180	840/672**
No. of blood bags stored 4R9953	-	-	-	-	-
Performance					
LN2 Capacity L	1.5	3.6	4.3	12.3	6.8
Static Evaporation Rate L/day	0.19	0.26	0.20	0.09	0.35
Unit Dimensions					
Neck Opening in. (mm)	1.40 (35.0)	2.75 (70.0)	2.00 (51.0)	2.00 (51.0)	3.81 (96.7)
Overall Height in. (mm)	13.5 (343)	18.4 (468)	19.4 (492)	25.7 (652)	25.0 (635)
Outer Diameter in. (mm)	7.25 (184)	8.70 (222)	8.70 (222)	14.50 (368)	14.50 (368)
Canister Height in. (mm)	5.0 (127)	11.0 (278)	11.0 (278)	11.0 (278)	11.0 (278)
Canister Diameter in. (mm)	1.20 (31)	2.62 (67)	1.81 (46)	1.50 (38)	3.20 (80)
Weight Empty lb. (kg)	6.0 (2.7)	11.0 (5.0)	13.0 (5.9)	30.0 (13.6)	28.4 (12.9)
Weight Full lb. (kg)	8.8 (4.0)	17.0 (7.7)	20.6 (9.3)	52.0 (23.6)	38.4 (17.4)

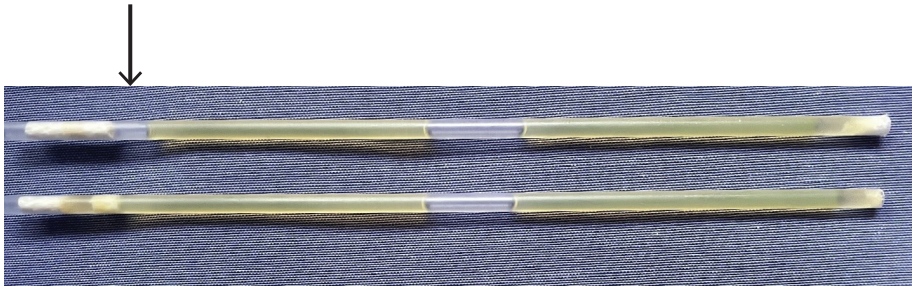

Figure 2. Right versus wrong filling of a straw before freezing. Upper straw has incomplete filling (air space close to the cotton plug; see arrow) compared to lower straw where semen is pulled up correctly to harden the polyvinyl powder between 2 cotton plugs, therefore avoiding entrance of liquid nitrogen

Author regularly weighs a shipper before it leaves to confirm that it is fully loaded. Author also weighs a dryshipper when it arrives in the clinic. It is highly recommended that semen arriving in a dryshipper be transferred to a wet tank upon arrival. Author has recently experienced the loss of shipped semen due to a sudden malfunction in holding time of a dryshipper that had been fully loaded and was working well in the past. If the receiving clinic would have transferred the semen upon arrival and not have left the semen in the dryshipper for several days until the planned insemination, the semen could have been saved. Author always transfers incoming semen from the dryshipper to a wet tank in her semen bank and records the weight of the arrived dryshipper before sending it back. Again, if the temperature is around –130°C or warmer, there may be a problem with recrystallization of the received semen. Author often observed personnel topping up the dryshipper with liquid nitrogen upon arrival, but then it is too late to know if semen arrived at a critical temperature or not.

Author has examined the bottom temperature using a thermocouple instrument (Reproduction Resources, WI, USA) of 2 dryshippers each weighing 7.7 kg when fully charged (vapor only). First dryshipper (empty weight considered 4.5 kg) had a temperature of –130°C and weighed 5.2 kg whereas the second dryshipper (empty weight considered 5.0 kg) had a temperature of –99.8°C and weighed 5.5 kg, suggesting that a weight difference of 300 grams resulted in a major difference in temperature when close to the empty weight. Again, despite the presence of vapor, the author measures the bottom temperature of all dryshippers that arrive close to their empty weight with semen in it and transfers the semen to a wet tank upon arrival.

Most often, frozen straws or pellet vials are attached to metallic canes and easily retrieved from a dryshipper. It is, however, advisable to verify that all semen received correlates with the enclosed paperwork. The canister should be inspected if semen is missing as frozen straws or vials may have fallen off the cane to the bottom of the canister during transport. Also, some European countries have a different way of packaging semen straws. Sometimes, there are semen straws left loosely in the bottom within a big plastic goblet covered with cottonwool. In other cases, straws are not in goblets on canes but in cassettes in the very bottom of the canister.

Thawing but not 'killing' sperm

Thawing semen appears very easy but may be tricky. In a few seconds, a few straws of semen and thousands of dollars that client paid can be wasted. Once a straw is removed from the water bath, water must be wiped off from the straw (water is spermicidal) before emptying the thawed semen into a tube where all semen is pooled. Different methods of semen freezing may demand different protocols for thawing. So, it is crucial to keep thawing instructions with the inventory or inquire about thawing instructions from the shipper to have them available when insemination is performed. Even a trained person can experience problems when thawing semen. Some semen straws may 'explode' upon thawing and the semen straw may end up in the corner of the room. This is often due to incorrect filling technique prior to freezing. To apply a good freezing technique, it is essential to moisten the cotton end of the straw when filling the straw with the semen, so that no air space is possible between the media and the cotton plug because such air space draws liquid nitrogen into the straw (Figure 2). Warming expands liquid nitrogen, the pressure created pushes cotton plugs into water and propels the straws out of water bath. Although metal or glass balls are good sealing methods for straws, the author has encountered most 'explosions' in straws closed with metal or glass balls. Author, therefore, always keeps frozen semen straws in a styrofoam dish with liquid nitrogen before thawing to examine how straws are filled to avoid problems. Author usually cuts straws off at the ball-sealed end under nitrogen to prevent any pressure problems to avoid semen loss. Similarly, if the straw is fully filled without any visible air bubble within the straw and well-sealed at both ends; the author prefers cutting the sealed end under liquid nitrogen. Straws are then thawed with an open end, as done with straws frozen by the CLONE technique. Due to pressure changes in such incorrectly filled straws during thawing, cotton plugs are pushed out and water can enter the straw from the bottom and semen is wasted. Once the sealed end is opened, this does not occur. Care must be taken that no thawing water enters the upper, opened end in such cases.

Thawing pellets of frozen semen in a whirl pack is probably the easiest method. Yet even here attention to detail is important. The whirl pack should be undamaged so that water cannot enter and mix with semen. After the semen vial is removed from the tank, it is crucial that all liquid nitrogen within the vial is allowed to evaporate prior to placing the pellets into the whirl pack. Otherwise, any remaining liquid nitrogen kills sperm in the water bath. If thaw medium is necessary to thaw the semen, it must be ensured that the thaw medium had enough time to warm up to the desired thawing temperature. During transcervical or surgical insemination procedure, it is best to wait to thaw the semen until the uterus is entered. In general, the author never performs any transfer or thawing of frozen semen without having a shallow styrofoam dish with liquid nitrogen in proximity to keep semen safe until ready to thaw.

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Conclusion

This paper is meant to emphasize the importance of paying attention to details when working with frozen semen. Simple techniques and preventive steps can ensure that avoidable damage to frozen semen was not the reason for pregnancy failure.

Conflict of interest

None to report.

Acknowledgement

This article is dedicated to late Professor Dr. W. Leidl (1925-2023) who taught theriogenology while the author was a veterinary student at the University of Munich, Germany. His excellent lectures and rigorous training inspired the author to become and be an enthusiastic theriogenologist for the past 40 years.

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