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Theoretical aspects of canine semen cryopreservation

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Abstract

Changes in canine sperm cells during freezing and thawing can cause damage to the cells resulting in cell death. No standardized freezing or thawing method appears to be ideal for all dogs and all ejaculates, because intrinsic variations in properties such as osmotic sensitivity between sperm cells from different dogs and ejaculates makes the cellular response to cryopreservation unpredictable according to the normal physics of cryobiology. Research in canine semen cryopreservation is difficult because the low ejaculate volume makes multiple comparisons from a single ejaculate difficult. True fertility data is also very limited on cryopreserved canine ejaculates. Despite this, the cottage industry that has evolved to cryopreserve dog sperm has been very successful using empirically derived methods that accommodate most ejaculates. Therefore, the practitioner must follow the recommendations supplied by the freezing center to achieve the best potential results.
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1. Introduction

Few small animal theriogenology practitioners will ever have substantial involvement with canine semen cryopreservation. For most, thawing cryopreserved semen and evaluating motility of the sample before insemination are the most technical procedures

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that will be undertaken with cryopreserved semen. Therefore, a detailed review of all the theoretical aspects of canine semen cryopreservation is not essential. In contrast, the goals of this manuscript are to provide the small animal practitioner with the basic principles and procedures of semen cryopreservation, highlight the challenges in doing research on canine semen cryopreservation, and provide recommendations regarding thawing of cryopreserved semen.

2. Principles of cryopreservation

During the cryopreservation process, cells are exposed to cryoprotective agents such as glycerol, ethylene glycol or dimethylsulfoxide in an extender. The addition of 'cryoprotectant' agents exposes the cells to a hypertonic environment; this causes the cells to initially shrink, but they regain their normal volume as the cryoprotectant enters the cells. Cooling also changes the cell membrane. During freezing, cells again shrink as water flows out of the cell and ice forms in the extracellular space. Depending upon the cooling rate, ice may form inside the cell as well. Formation of intracellular ice usually results in damage to the cell membrane; it loses its semi-permeable properties, resulting in death of the cell. During thawing, the cells undergo similar changes in volume as water moves back into the cell. The thaw rate and cell survival are dependent upon the cooling rate used. As stated by a researcher considering the difficulties in semen cryopreservation of other species compared to the bull, 'it causes one to marvel at successful survival rather than to despair over the difficulties of its implementation to the preservation of a variety of other sperm cell types' [1].

3. A simple cryopreservation protocol

Many different methods of canine semen cryopreservation have been empirically formulated. For example, a simple, empirical freezing method developed at Louisiana State University has been published [2]. After semen is collected and evaluated, it is diluted 1:1 using a commercially available semen refrigeration extender (Refrigeration Media: test yolk buffer (TYB)-9972; Irvine Scientific, Santa Ana, CA, USA) and centrifuged for 10 min at $900 \times g$. The sperm pellet is re-extended to 400×10^6 cells/mL and placed in a refrigerator (5 °C) for 1 h. Next, a commercial freezing extender containing 12% glycerol (Freezing Medium: test yolk buffer (TYB) with glycerol-9971; Irvine Scientific), also cooled to 5 °C, is added (1:1) to the cooled semen sample (final concentration, 200×10^6 cells/mL with 6% glycerol). In a 5 °C cold box, 0.5 mL straws are filled by aspiration and the end is heat-sealed. The straws are then placed on a screen that is attached to a 3 cm thick Styrofoam frame, which is floating in liquid nitrogen. After 10 min, the straws have cooled to −100 °C and they are plunged into the liquid nitrogen. Straws are subsequently thawed at 50 °C for 10 s. If the pre-freeze sample had good motility and morphology, this protocol usually results in excellent post-thaw motility.

4. Other cryopreservation protocols

There are many other protocols, with differences in extenders, cryoprotectant agents, concentrations of cryoprotectants and semen, cooling rates, freeze methods (vapor or programmable freezers), freeze rates, storage methods (straws versus pellets), thaw rates and thaw extenders [3,4]. However, there are no standards within the industry regarding cryopreservation methodology, reporting sample concentration, recommended breeding doses, or post-thaw semen viability. Furthermore, an ejaculate may contain relatively few spermatozoa; it is not uncommon to have only one insemination dose from a single ejaculate. This makes fertility comparisons within a single ejaculate impossible. Under field conditions, few dogs will have 50–100 ejaculates cryopreserved and inseminated into 100 bitches using similar breeding techniques in order to provide a meaningful assessment of fertility.

5. Other factors that affect fertility

In addition to differences among males and cryopreservation protocols, there is the female aspect. In that regard, there is the inherent fertility of the female, the variability of each female's estrous cycle, the insemination method (surgical, 'Nowegian' transcervical, 'New Zealand' transcervical, vaginal), the timing method (LH and/or progesterone), differences among laboratories in hormone concentrations, timing of insemination (inseminating on days 5–6 after LH versus inseminating based on progesterone concentrations), the number of inseminations, and the number of cells in the insemination (or total normal motile cells inseminated). With all these variables, it is difficult for the practitioner to feel confident about the individual cryopreserved sample that is to be used. Despite all these differences and problems, it is remarkable that the industry has been relatively successful and there is increasing demand for cryopreserved canine semen.

6. Research challenges

Researchers are constantly studying various aspects of canine semen cryopreservation in order to improve the final result, puppies. There are many challenges. Dogs are expensive to purchase and house. The canine ejaculate contains relatively small numbers of spermatozoa, which limits performing multiple treatments on a single sample and makes it difficult to obtain sufficient insemination doses to measure fertility. Regardless, researchers are actively investigating extenders, cryoprotectants, freeze rates and thaw rates.

7. Effects of extender

Many different extenders have been developed for many species. Extenders help stabilize the cell membrane. The extender, usually with a cryoprotectant added, changes

the solution osmolarity and results in temporary cellular dehydration. The osmotic sensitivity of the cells is very important in the cryopreservation process. While the effects of osmotic stress have been investigated in other species, there has been little information regarding the effects of osmotic stress on canine spermatozoa during cryopreservation. A study was done by a collaborative group in Louisiana to investigate these effects [5]. Five ejaculates each were collected from three dogs at Louisiana State University School of Veterinary Medicine. Semen was centrifuged for 5 min at $200 \times g$, the supernatant discarded, and the pellet was re-extended in either a stallion skim milk extender or an egg yolk–citrate extender for the sodium chloride (NaCl) study, skim milk only for the monosaccharide study, or egg yolk–citrate or electrolyte free medium for the cryoprotectant studies. The extended semen was placed in a transport container and shipped overnight to the Audubon Center for Research on Endangered Species where the osmotic sensitivity studies were performed. Osmotic sensitivity was tested using NaCl at 22 °C and 290, 500, 700, 1000 or 1500 mOsm, or monosaccharides (glucose, galactose or fructose) at 4 °C at 290, 500, 700, 1000 or 1500 mOsm, or cryoprotectants (glycerol, ethylene glycol or dimethylsulfoxide) at 4 °C at concentrations of 0.3 or 0.6 M. Analysis of motility was estimated by a trained observer at 200× magnification and membrane integrity was performed using a double-fluorescent live/dead stain at 400× magnification with a fluorescent microscope. Data are summarized in Table 1.

The transported semen decreased in motility in NaCl at 500 mOsm in the egg yolk–citrate extender. However, it did not lose motility in samples from two of three dogs in the skim milk extender. A definite male-to-male difference in motility was noted after shipping in skim milk extender. Membrane integrity decreased at 500 mOsm in both egg yolk–citrate and skim milk extenders. However, in the skim milk-extended semen, there

Table 1
Motility and changes in membrane integrity of canine sperm after dilutions in NaCl, exposure to cryoprotectants, or monosaccharides

	Extended in egg yolk–citrate	Extended in Kenney skim milk
Motility in 500 mOsm NaCl	↓ 3/3	↓ 1/3 (male difference)
Membrane integrity in 500 mOsm NaCl	↓ 3/3 (1/3 differed between 290 and 500 mOsm)	↓ 3/3 (male difference seen)

	Extended in egg yolk–citrate	Extended in G-BSA
Motility in 0.3 M dimethylsulfoxide, 0.3 and 0.6 M ethylene glycol and glycerol	↓ 3/3	↓ 3/3
Membrane integrity in 0.3 M dimethylsulfoxide, 0.3 and 0.6 M ethylene glycol and glycerol	↓ 3/3	↓ 3/3
Motility in 0.6 M dimethylsulfoxide	↓ 2/3	↓ 3/3
Membrane integrity in 0.6 M dimethylsulfoxide	↓ 3/3	↓ 3/3

	Monosaccharides (glucose, galactose, or fructose; semen extended in skim milk only)
Motility in 500 mOsm	↓ 3/3
Membrane integrity in 1000 mOsm	↓ 3/3

appeared to be the same male-to-male difference in membrane integrity. The transported semen decreased in motility in monosaccharides at 500 mOsm. Motility decreased further as the osmolarity increased and was completely absent at 1500 mOsm. Membrane integrity decreased at 1000 mOsm in monosaccharides. Motility was decreased in semen from all dogs in 0.6 M dimethylsulfoxide. The egg yolk–citrate-extended semen resulted in better overall motility in one dog, but not the other two. Membrane integrity was not affected by treatment, even though samples exposed to 0.6 M dimethylsulfoxide had lower motility. Since dimethylsulfoxide has a lower molecular weight, it should enter the cell more readily than the other cryoprotectants and cause less osmotic damage, indicating that there may be a direct toxic effect of the dimethylsulfoxide on the cell. Perhaps a shorter exposure time may be beneficial when freezing. Motility, but not membrane permeability of the semen transported in egg yolk–citrate, was more affected than that transported in skim milk. This could be because of long-term incorporation of lipoproteins and cholesterol into the plasma membrane. Conversely, the egg yolk–citrate extender seemed to have a protective effect when tested with dimethylsulfoxide. This study showed that canine sperm cells are sensitive to osmostic stress, but tolerate shrinking.

8. Cooling rate

As the osmotic stress causes changes in the cell water volume, the cooling rate of the sample affects the membrane permeability and cell survivability. Cooling rates that are too slow or too fast cause intracellular ice formation and result in cell death. Different cell types have different optimal cooling rates, because of different membrane permeability. A novel method was used to measure canine cell permeability, and to calculate the theoretical optimum cooling rate [6]. Eight Beagle dogs had ejaculates collected and pooled. The semen was extended 1:1 with a skim milk extender and then transported to the bioengineering department. One of two (glycerol at 0.5, 3.0 and 6.0% or dimethylsulfoxide at 0.5 or 3.0%) or no cryoprotectants were used. Sperm cells at a concentration between 200 and 500 $\times$ 10^6 cells/mL were cooled. The membrane permeability was measured using differential scanning calorimetry; this measures heat release during freezing of living and dead cells (the difference in heat release is correlated to water transport). Using the canine sperm cell modeled as cylinder, membrane permeability parameters are determined and used to calculate intracellular water loss. These predictions are then used to estimate the amount of intracellular water with no intracellular ice formation. Using all these data, an ideal cooling rate is determined. The theoretical optimum cooling rate was calculated to be between 10 and 30 ^{o}C/min, which is close to the empirically derived cooling rates.

The physics of cryobiology explains what happens, but the variability in response among dogs cannot be explained by calculations and equations. Anyone who has cryopreserved canine spermatozoa knows that, even if pre-freeze motility and morphology are similar, some dogs have semen that survives cryopreservation better than others. This may be caused by genetic differences in the cellular membrane that results in differential membrane permeability altering water movement. The altered water movement may result in intracellular ice formation and cell death.

9. Semen thawing

The thawing procedure is of critical importance when using cryopreserved semen. The involvement of the practitioner in the cryopreservation process is typically limited to semen thawing. Furthermore, the practitioner determines when to inseminate and performs the insemination procedure. The optimal thaw rate of cryopreserved epididymal sperm cells is related to the cooling rate. Spermatozoa cooled at 10 °C/min and warmed at 37 °C for 30 s (rapid warming) had better post thaw motility than spermatozoa thawed at slower rates if cooled at slower or warmer rates [7]. There was also significant variation among dogs, again indicating that there is no ideal universal system [7]. It should be emphasized that some semen freezing companies require the addition of a diluent during the thaw procedure. The procedure outlined by the company that cryopreserved the sample should be followed closely.

The cryopreservation of canine spermatozoa is still mostly a 'cottage industry'. Methods to cryopreserve spermatozoa have been derived empirically and are tailored to accommodate most dogs. However, spermatozoa from a particular dog may not endure the cryopreservation process as well as another. Since most freezing centers have a very limited selection of extenders, cryoprotectants, cooling rates and freeze rates, it makes it difficult to find the best cryopreservation technique for any individual dog. Even if a complete range of extenders, cryoprotectants, cooling rates and freeze rates were available, the small volume of the dog ejaculate would make multiple cryopreservation protocols time-consuming and expensive.

The best advice for practitioners when using cryopreserved semen is to follow the freezing center's guidelines for thawing. This will help to maximize the theoretical fertility of the sample being inseminated. Do not use a 'standard' method for samples cryopreserved in different places using different methods. If the practitioner handles the cryopreserved semen well and motility is poor, it must be remembered that there are many other factors that could be responsible for the poor motility.

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