Freezing semen, thawing semen from a practitioner's perspective

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In domestic ruminants, reproductive efficiency is the driver of economic returns. Ideally a cow, ewe, etc. produces offspring every 12 months.[1,2] Beef cattle tend to have a per service pregnancy rate of about 58 to 60% which over three service intervals should yield an overall pregnancy rate of over 90% (the reader should realize that being pregnant does not necessarily lead to a full-term parturition event). Producers might not realize this and grow disappointed with an AI pregnancy rate that is, let us say, 60%, because they assume a whole breeding period pregnancy rate with a bull is due to single, not single plus multiple, services.

Of factors contributing to a successful artificial insemination event, the following points all factor in:

1. Nutrition
2. Management (executing protocols and adequate facilities)[4]
3. Semen or embryo quality
4. Capability of practitioner or technician

Of these points, nutrition has the largest impact on reproductive success.[3] A veterinary practitioner needs to understand and be able to communicate to a producer the importance of digestible NDF and protein and where this sits to microbial production of amino acids, volatile fatty acids, vitamins, etc. It must be said that trace mineral and vitamin deficiency, will certainly lead to poor reproductive outcomes as well; in this there is an opportunity for a practitioner to gather samples via liver biopsy and serum sampling for trace mineral and vitamin adequacy. This information can form a backbone for nutritional consultation.

Poor outcomes from an AI/ET protocol can be due to many reasons. An oocyte takes about 90 days to progress from a primordial follicle to an ovulatory follicle. During this period, events good and bad are noted and will reflect in the ability of the oocyte to fertilize and eventually result in a live calf. The same timeline (a bit shorter – at 60 days [about 2 months] in bulls) exists for males as well with similar effects. To sum this part up – management counts…

Cryopreservation of spermatozoa has been utilized since the late 1940s when Polge et al[6] discovered, by accident, the use of glycerol as a cryoprotective agent in cell cryopreservation. Cell cryopreservation utilizes a hyperosmotic cryoprotectant agent (which is also inert in terms of cell function) such as glycerol, ethylene glycol, DMSO, sucrose, galactose and egg yolk to remove water from cytoplasm prior vitrification. Intracytoplasmic water will form ice crystals and damage organelles, etc.

Cryopreservants also help in membrane stabilization.[8] It is important to utilize an agent such as 5-15% glycerol or 1.5M ethylene glycol (9.3%) to pull water out of the cytosol and replace it with the osmotic agent or simply pull water out of the cytosol (sucrose and galactose cannot cross cell membranes) for the cell being frozen to survive post thaw. After an initial equilibration period the extended semen or embryos in cryopreserving solution are placed in subfreezing temperatures (the methodology varies depending on what is being frozen).

Extenders for freezing semen are placed in two categories – synthetic and egg yolk and then as one-step versus two-step. Synthetic extenders contain lecithin, low-density and high-density lipoproteins, etc. To stabilize spermatozoa membranes, no animal-derived products are utilized. Egg yolk extenders use egg yolk to do the same function. One-step extender has glycerol concentration at the level necessary for cryopreservation premixed (at 5-7% glycerol). Two-step extenders have two identical phases except phase 1 has no glycerol in it, phase 2 is the same extender mixture with 14% glycerol added. With one-step extension, the sperm quantity is determined and the final quantity per dose is determined. Then the appropriate quantity of one-step extender is added to the semen. The extended semen, once cooled and equilibrated, will be drawn up into straws and frozen.

To freeze semen with two-step semen extender, one extends the semen using phase 1 with half of the volume needed for final volume and antibiotics (usually gentamicin, tylosin, lincomycin and spectinomycin) and cool the semen down. Once the semen is cooled down to less than 20 °C, phase 2 is added (same temp as semen) to QS the semen to desired volume. This is cooled down for several hours to 4 °C, allowed to equilibrate for a couple hours, pulled up in straws and frozen. What cryopreservant is used depends on bull/ram/buck preference, the person freezing and market specification (export, etc.). I prefer one-step synthetic extenders such as Optixcell from IMV.[15] Others prefer tris/egg yolk extenders which can be purchased pre-mixed or made on site with the addition of egg yolk being necessary.[16,17] Some males do better with one cryopreserving agent vs. another; other males seem to have no preference.

In the freezing process, the extracellular water begins to form into pure ice crystals. This process is called seeding. Seeding with pure ice crystal formation is temperature- and cryopreservant-dependent. In embryos in an ethylene glycol or glycerol solution, seeding is initiated at -3 to -6 °C by touching the side of the straw above and/or below the column containing the embryo with a piece of metal or cotton tip dipped in liquid nitrogen. With embryos, the cryopreservant fluid will self-seed at -9 °C, but if self-seeding at less than -6 °C occurs, the ice crystals might not be pure water, so the unfrozen solute will not increase osmolality to drive embryo dehydration in the manner desired. An embryo, at the point of vitrification, will be about 60% dehydrated. With semen cryopreservation, seeding is not actively initiated, rather the straw, when placed 3-6 cm over liquid nitrogen (the temperature will be about -100 °C) undergoes something between self-seeding, then vitrification and simply vitrification. Probably one reason for the movement in the AI industry from 0.5 ml straws to 0.25 ml straws is the freeze gradient from outside to center is more uniform. Once the semen straw has suspended over the liquid nitrogen for 10 minutes, the straw will be completely frozen. Semen freezes more rapidly than embryos but the equilibration and solution cool down is more prolonged than it is with embryos, probably due to

stabilized membranes prior to freezing; membrane stabilization has to be considered due to the inability of spermatozoa to undertake any organelle or membrane repair.[8]

The process of freezing embryos or spermatozoa is taxing on cell function; depending on cell quality/viability, a portion of the embryo cells or spermatozoa will die with cryopreservation. The amount of cell loss from cryopreservation is correlated to embryo grading used by AETA and IETS. With spermatozoa, freezing will sometimes kill half or more of the spermatozoa. Because of the loss of some percentage of cells due to freezing, one must attempt to freeze only the best samples. With conventional (in vivo-produced) bovine embryos one can freeze #1 IETS grade (> = 85% of cell wall appears normal) late morulae and early-stage blastocysts with pregnancy rates approaching or exceeding artificial insemination using either ethylene glycol (direct thaw) or glycerol as a cryopreservant. Frozen thawed (non-gender selected) semen will give comparable results to fresh semen if post thaw semen meets minimum standards is utilized.

Some skills need to be mastered to successfully freeze semen. First, one needs to learn to make a good slide for semen evaluation. The process is simple. Morphology and motility assessments are from Society for Theriogenology:[7]

A. Use only new slides, clean each slide prior to use, have a slide warmer.
B. Cover slips (try to keep these in a low humidity environment as they stick together when in elevated humidity).
C. Have a diluent (I use saline – 5 ml with 2 drops of eosin nigrosin solution from SFT) – warm to 34-36 °C.
D. Have a 5-10 UL pipettor with disposable tips.
E. A good microscope – I prefer a phase contrast with dry phase 40x and 100x oil objective lenses.
F. I want to have about 20-30 sperm per 400x field and have a single layer of cells (so spermatozoa aren't swimming in and out of focus and they can move without hitting each other).
G. A water bath at 35-36 °C.
H. A floating semen rack.
I. Either a hemocytometer or a miniCASA such as iSperm.
J. A 10-15 gallon Styrofoam cooler.
K. Preprinted straws, syringe with straw adapter to fill straws (I use a cut tomcat catheter).
L. Extender, in water bath.
M. Liquid nitrogen.
N. A storage dewar.
O. Canes, goblets, tabs.
P. Hemocytometer or a CASA or miniCASA.
Q. 50 ml centrifuge PET tubes, sterile.
R. Extender (one- or two-step with antibiotic).[15,16,17]

Determining sperm concentration

To determine sperm concentration, several methods have been used.[9] For the private veterinary practitioner, the most practical tools for sperm concentration determination are a hemocytometer and a miniCASA. In either case, microscopy is necessary to determine sperm morphology, and in the case of hemocytometer use, progressive motility. The use of hemocytometer is described in **https://animal-reproduction.org/journal/animreprod/article/5b5a6043f7783717068b4671.**[10]

Total spermatozoa quantity is calculated by amount of semen plus extender volume x spermatozoa concentration per ml. Once this quantity is found and the sperm quality is at or above adequate (> 80% progressive motility and > 70% normal morphology) then one can calculate the number of straws of semen to be frozen. A minimum of 35% of the frozen thawed spermatozoa should be progressively motile. The total amount of sperm per straw at a minimum progressive motility of 35% varies quite a bit depending on author.[11-13] When this author freezes semen, he wants to have 10 to 20 million progressive motile spermatozoa per straw; this means that a total 45 to 50 million spermatozoa are in an inseminating dose. Reducing spermatozoa dose will still yield acceptable results. However, lowering the dose makes timing of insemination to ovulation more critical. This is seen with use of gender selected semen, in part due to changes that occur to the spermatozoa in the gender selection process where a degree of capacitation occurs.

Method for freezing sperm in clinic

1. Place straws, PVA powder, semen rack in refrigerator × 1 + hours.
2. Fill large Styrofoam container with 4-5 cm liquid nitrogen, cover container.
3. Place chilled semen in a same temperature shallow water bath.
4. Agitate semen with a straw (do this every other straw that is filled).
5. Pull up half of a straw of semen using syringe and adapter, then pull up 0.5 cm air then complete the fill so straw plug is saturated.
6. Push open end into PVA powder to seal; wipe off excess powder.
7. Place on rack holding the cotton plug end.
8. When done with straw filling, place rack on liquid nitrogen (it will float).
9. Wait 10 minutes then flip rack placing straws in nitrogen.
10. Pull out a straw, thaw in 35 °C water, place sample on slide, coverslip and check under microscope or iSperm.
11. Let the sample "wake up" for 5-10 minutes.
12. If quality is acceptable, then put straws in goblets in identified canes, place in tank.
13. If semen quality is less than 35% progressive motility, I would discard the sample.

The key is to be quick and keep temperature stable and 4-5 °C.

Another option that practitioners can offer for cryopreservation of bovine semen is to work with one of the smaller bull studs to send them extended chilled semen to be processed and frozen at the bull stud facility. I have done this with success on many occasions with Hawkeye Breeders Services in Adel, Iowa. The semen requirements (> 80% progressive motility and > 70% normal morphology) are the same as for freezing at one's clinic. The advantage for the practitioner is that storage of client frozen semen inventory and filling sale orders can be time consuming. If sending out semen for cryopreservation I will first contact the bull stud and ask what sort of cryoprotectant they would like for me to use. Make sure, if using a one-step product that you already have, that they have that same product in stock on their end so they can further extend the semen to final volume. I collect the semen with an electro ejaculator. I try to perform two collections with an hour or so in between collections. Each collection is packaged separately and identified. I do not quantify the spermatozoa on sent out semen; I record ejaculate volume. After semen evaluation I place the tube with the sample in a 35 °C water bath and extend the semen with twice the semen volume of extender (at 35-36 °C) to be utilized. I then will wrap the vial with a paper towel, then wrap with bubble

wrap and then place next to a frozen ice pack in a chilled semen shipping container such as a "Semen Shipping Kit" from Reproduction Provisions.[14] These shippers are reusable. The semen is shipped via an overnight shipping service and frozen the following day.

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