Collecting and processing semen from cervids, sheep and goats

Manoel Tamassia
New Jersey Department of Agriculture, Trenton, NJ

Abstract

The ability to freeze semen from domestic species has provided the farming community with an inexpensive way to preserve and propagate superior genetics. During cryopreservation sperm will endure severe physical and chemical challenges. There is significant difference in sperm from different species. The sperm shape, size and lipid composition will affect its ability to survive the freezing and thawing process. An understanding of the freezing requirements for the different species is necessary to increase sperm survival. The purpose of this review is to present to the practitioner the basic information necessary for the successful cryopreservation of sperm in small ruminants and deer.

Keywords: Sperm, cryopreservation, reproduction, small ruminants

Introduction

The purpose of this article is to provide important background information for understanding the process of cryopreservation of semen in small ruminants and deer. Differences in semen and cryopreservation techniques exist among domestic farmed animals and several reviews with more detailed information are available.[1,2]

Semen collection

The use of an artificial vagina (AV) is the preferred method of semen collection since the technique is successful in a high percentage of bucks and rams and a representative ejaculate is obtained. Semen collection with an AV should be made during attempts to breed a mount, ideally an estrual female. Many males, especially if used to hand breeding, will mount a nonestrual female if she is sufficiently restrained to prevent rearing and limit lateral movement.

Semen samples can also be collected using electroejaculation. Several types of electroejaculators and probes are available commercially. Independent of the model used, the rectum of the animal should be emptied prior to collection. A ram probe can be used to collect bucks. The desired response is penile extension followed by ejaculation. One main advantage of electroejaculation is that it can be used in the absence of a mount female or when the male is unable or unwilling to mount.

Semen from farmed deer is obtained by using either an AV or electroejaculation[3]. Most stags are not tame or trained to be collected using an AV. Electroejaculation must be used when the objective is to repeatedly recover samples from selected males. Electroejaculation must be performed in combination with effective and secure anesthesia (http://www.ivis.org/special_books/Heard/caulkett2/IVIS.pdf). Electroejaculated semen can have a different composition when compared with semen collected by more natural methods (e.g., AV). This difference is due to differential stimulation of seminal glands by the electroejaculation probe resulting in high variability between ejaculates.[4]

Alternatively, semen can be collected post-mortem by recovery of epididymal spermatozoa, from hunt harvested animals.[5-9] These techniques have been tested not only in deer, but also in many other wild ruminant species.[4]

Semen evaluation

The principal objective is to assess the number of motile, morphologically normal spermatozoa in the ejaculate prior to freezing. Volume of semen, sperm concentration, proportion of motile sperm and spermatozoa morphology must be measured. Values must be determined with precision to provide useful information to be used in the dilution and freezing process. Of these variables sperm motility is the most difficult and inaccurate measurement. Motility should be estimated on a warm slide (35° C) using a good microscope, preferably equipped with phase-contrast illumination for an accurate evaluation. Stained slides of semen can be prepared for morphological evaluation of sperm. A common stain is eosin-

nigrosin as it also permits the assessment of viable (unstained) sperm. Semen should have at least 70% normal sperm cells with good motility and concentration. Normal sperm concentration varies from animal to animal with a normal ejaculate containing at least a billion sperm cells in sheep and goats. The estimated semen output from red deer collected by electroejaculation is more than three billion sperm/ejaculate. In the same study, 2.5 billion/sperm were collected post mortem.[10] Semen concentration will vary with the age of the animal, method of collection and health status.[10] In some deer semen collection, using electroejaculation, one may observe a thick gel fraction. This should be collected separately from the sperm-rich portion as sperm will stay trapped in the gel.[11,12]

Sperm concentration can be determined accurately with the use of a hemocytometer. Other methods of counting cells are also available and will not be discussed here. The total number of sperm in the ejaculate can be estimated by multiplying the number of sperm/ml (concentration) by the volume of the ejaculate. This product multiplied by the percentage of normal sperm gives the number of normal sperm in the ejaculate.

Seminal plasma

For years, the only available model used for successful cryopreservation of small ruminant semen was the technique used for cattle. However, this technique did not work well with goat semen. The success was limited due to the deleterious interaction between the bulbourethral gland secretion and egg yolk used on the semen extender. This was known to be caused by an egg yolk coagulating enzyme.[13] This enzyme was shown to hydrolyze egg yolk phospholipids into lysophospholipids such as lysolecithins, which are toxic to spermatozoa.[14] This problem is not seen with bull, boar, rabbit and ram semen.

To successfully freeze goat semen it is recommended to remove the seminal plasma if egg yolk or milk extender is being used. However, seminal plasma secretions appear to be beneficial to preservation of sperm integrity during freezing in the absence of egg yolk. The removal of the seminal plasma is carried out by dilution of ejaculated sperm in an isotonic buffer followed by centrifugation (washing method). Washing increases the storage duration of chilled goat sperm and also improves the survival of spermatozoa after freezing and thawing.

Sperm dilution or concentration

Semen samples from farm animals have been diluted prior to freezing based solely on volume (v/v) or to a specific final sperm concentration. The second method being preferred as it provides uniformity to the samples necessary for commercialization of straws. An insemination dose should have forty to one-hundred million viable sperm. This concentration has been reported to achieve acceptable pregnancy rates in small ruminants. The number of sperm/insemination dose can be reduced drastically if the semen is to be deposited deep into the uterus.

Sperm concentration can be determined accurately with the use of a hemocytometer. The hemocytometer is a device used to count red and white blood cells but it can also be used to count sperm. Sperm must be diluted with water or buffered formalin. The objective is to immobilize the sperm cells and to dilute them sufficiently so that you will count 100 to 400 sperm cells in the five squares. The central counting area of 25 large squares has an area of 1 square mm, and the coverglass rests 0.1 mm above the floor of the chamber. Thus, the volume over the central counting area is 0.1 microliter. In other words, to calculate the number of sperm/ml of original sample one must calculate the mean number of sperm counted for each chamber (i.e. for each of the central counting areas - 25 squares). Then multiply the mean obtained in the two chambers by 10,000 to obtain the number of cells/ml of diluted sample. Finally, multiply the count obtained in by the dilution factor.

Once the concentration of sperm in the ejaculate has been determined, the sample will need to be diluted with extender or concentrated (through centrifugation) to adjust the insemination dose to fit into one straw or cryovial. The most common diluents used to extend sperm contain either non-fat dried skin milk or egg yolk.

Cryopreservation

Cryopreservation of sperm is a simple process that involves the complex balancing of many reagents with the objective of obtaining satisfactory results (viable sperm that can fertilize the oocyte and result in a normal pregnancy). The process can be intuitive but knowledge of sperm physiology for each species is important for success. Semen extenders are a complex mixture of reagents. The use of diluents in cryopreservation of sperm is necessary as they provide a source of energy, protect the cells from temperature related damage and maintain an environment where the sperm can survive. Egg yolk and skim milk in semen extenders exert a protective effect against cold shock upon chilling or freezing of spermatozoa.

Egg yolk is one of the most commonly used components of cryoprotectants used in sperm cryopreservation. In general a cryoprotectant medium contains a penetrating cryoprotectant (glycerol, ethylene glycol or dimethyl sulfoxide), a non-penetrating cryoprotectant (egg yolk or milk), a buffer (tris, HEPES), one or more sugars (glucose, lactose, fructose, saccharose or trehalose), salts (sodium citrate, citric acid), and antibiotics (penicillin and streptomycin). Proteins present in the extender (from milk, egg yolk or other protein sources present in the synthetic extenders) help to stabilize the cell membranes and act as a non-penetrating osmotic factor protecting sperm from cold damage during freezing. The beneficial effect of egg yolk can be attributed to the presence of phospholipids, cholesterol and low density lipoproteins.[15] Salts and sugars are added to maintain an isotonic solution. Sugars provide energy for the sperm cells and larger sugar molecules serve as osmotic regulators helping to dehydrate the sperm during the freezing process. Buffers are added to maintain a physiological pH level and should work over a wide temperature range. Antioxidants added or present in the egg yolk slow or prevent oxidation avoiding cell deterioration and death. Other antioxidants are added in some commercial semen extender formulas. Antibiotics are added to control bacterial growth from contamination that occurs during collection and through processing.

The freezing process is quite simple, i.e. extended sperm is exposed to cold and then plunged into liquid nitrogen. However the process is more involved than this simple description. The concentration and morphology of the ejaculate is determined and the final dilution is made (e.g. 200 million/sperm/ml). Addition of glycerol to the extender may be made in one step or in multiple steps (e.g. one step diluents and two step diluents). The diluted sperm sample is cooled to 5° C for at least 90 minutes and then frozen in pellets or straws. Freezing in pellets is accomplished with the aid of a block of dry ice (solid CO_2 with the surface temperature of -79° C) in which cavities have been carved. These holes are filled with 0.1 to 0.3 ml of extended semen and left to freeze for 2 to 4 minutes. Pellets are then transferred to labeled cryovials containing the expected volume necessary for an artificial insemination dose.

An alternative to freezing semen in pellets is freezing it in straws. Straws are easily labeled and can be easily and efficiently stored in semen tanks. Straws (0.5 or 0.25 ml) are loaded with semen after dilution and equilibration at 5° C for a period of time (varies according to the diluent being used) and placed on a rack over liquid nitrogen (3-4 cm) for 7-8 minutes (goat semen) and then plunged into liquid nitrogen. The semen concentration is adjusted prior to freezing to contain one insemination dose per straw.

Programmable freezers can also be used to freeze semen in straws. They are convenient to freeze large numbers of straws and allow precise controlling of the freezing curve. These machines can be programmed with multiple freezing steps for better results. This makes comparison of results during freezing very difficult. Some programmable embryo freezers may be used for this purpose.

There are several "recipes" for semen extenders listed in the scientific literature. For the practitioner we recommend the use of commercially available semen extenders. These formulas are available for a reasonable cost and eliminate the need to mix and keep reagents. They also avoid the need of having scientific equipment to mix, adjust pH, and have a reduced risk of contamination among other challenges. Most commercially available freezing solutions will require the addition of egg yolk and all handling should be done on a clean environment. Some commercial semen extenders have shown to keep sperm at room temperature for extended periods of times (several days). Refrigeration of extended semen at 5º C for several days is also possible. This allows semen to be collected on a farm and moved to the

clinic or laboratory for evaluation, extension and freezing. It also avoids the need to freeze semen increasing the number of breeding doses as it prevents sperm loss during the freezing and thawing process. Some commonly used commercial extender names are: Andromed ®, Bioxcell ®, Triladyl ®, Biladyl ®, BoviPRo Cryoguard ®.

Thawing

Quality of the frozen semen can be dramatically affected by the thawing method used. Some of these effects are due to the interaction of the sperm with the components of the semen extender and others are entirely related to the thawing process. The use of epididymal vs. ejaculated sperm also affects sperm survival after thawing.

Thawed frozen sperm should be evaluated for motility and longevity post-thaw. The best evaluation of success remains the pregnancy or offspring rate. Differences in post-thaw motility, viability and fertility may be attributed to the extender used, the cooling rate, the thawing method and the method of freezing (straw vs. pellets) among other variables. Thawing sperm is determined by the method used to freeze the sperm. Sperm pellets should be thawed in a dry test tube submerged in a 37º C waterbath for 15-30 seconds. Several temperatures have been used to successfully thaw semen frozen in straws. Straws were successfully thawed in 37º C water for 30 seconds to 2 minutes, or 40º C for 20 seconds or even the scalding temperature of 70º C for 7 seconds. Attention to temperature and timing becomes much more important as temperatures above 37º C are used. To avoid problems with fertility, one should always follow the thawing recommendations directed by the freezing institution.

Semen should be thawed immediately before insemination. Thawed semen should be used in its entirety once thawed. One should not try to refreeze the thawed semen as it is very likely to yield disappointing results. Split semen straws, i.e. using one straw to inseminate several females, is not recommended. However it has been done successfully. The rate of success for split straws will vary and is directly related to the quality of the thawed semen, the concentration, the site of semen deposition and method of insemination.

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