Trippin Two Eyed and Chillin'

Thomas Dalton Pate

Advisor: Heath King DVM, DACT

Introduction

Cryopreservation of semen has become increasingly common in the equine industry in recent years with many advantages stemming from its use. Such advantages include stallion availability no longer being limited geographically, no interference with competition schedules to coordinate stallion availability and mare estrous cycles, and preservation of valuable stallion genetics to ensure lineages carry on even after they are deceased [1,2,3]. With advances in cryoprotectants beyond glycerol and the help of improved processing techniques [3], we are now able to provide viable pregnancies with insemination doses as low as 50×10^6 spermatozoa [2]. These advances have benefited horse breed associations around the world and brought success to some stallions that would be considered “poor freezers” [3].

Widespread differences in stallion genetics, breed differences, sperm metabolism and other factors limit the ability to standardize a single protocol for freezing stallion semen [4]. Large variations exist amongst stallions with regards to how their individual semen can handle the freezing and thawing process. Sperm must be able to withstand cold shock, osmotic changes and ice crystal formations during the process [3,4,5]. Some have reported that roughly 20% freeze well, 60% freeze acceptably and 20% freeze poorly [4]. Others report that only 30-40% of stallions produce semen that is suitable for cryopreservation [5]. Since fertility and semen quality are rarely, if ever, taken into consideration when sires are selected for breeding, stallions that are collected with intentions of freezing semen should have careful attention paid to the quality of their semen [6]. Factors like morphology and motility both pre- and post-thaw should be taken into consideration.

Semen Processing

Once semen is collected, it is necessary to filter semen to remove the gelatinous portion and other debris that may be present. The gel-free semen is then diluted to a 1:1 ratio with a skim milk or casein based extender that has been pre-heated to 37˚C [3]. Centrifugation, typically performed at 600 x g for ten minutes, is commonly utilized to then remove the seminal plasma and concentrate the sperm cell in the ejaculate [3]. Studies looking at potential damage caused by standard centrifugation have led to other alternative methods. These include the use of a sperm cushion placed at the bottom of the tube during centrifugation, coupled with higher centrifuge speeds of 1000 x g and a twenty-minute duration [4]. The use of the sperm cushion and higher centrifuge force improves sperm recovery while preventing mechanical damage [4].

Freezing Extenders

Once centrifugation is complete, the supernatant is discarded and the remaining pellet must be resuspended into the appropriate freezing extender. Extenders help to stabilize the pH, neutralize toxic products of sperm metabolism, protect against thermal shock, inhibit bacterial growth with assistance of added antibiotics, maintain electrolytic and osmotic balance and supply energy to the sperm [3,7]. Egg yolks or skim milk are the primary components of extenders, usually with added ingredients like sugars and antibiotics. Egg yolk gained popularity due to studies supporting increased fertilizing ability as well as added protection from damage during cooling and freezing. This is thought to be due to the low-density lipoproteins, or LDLs, contained within the yolk [7]. It is thought that the LDLs form a protective membrane on the surface of the sperm by replacing the damaged phospholipids within the sperm cell membrane [7].

The major difference between the extenders used for freezing semen as compared to cooled semen is the addition of a cryoprotectant. During cryopreservation, ice crystals form in

the extracellular medium. This increases the osmolality of the unfrozen water, leading to a difference in the osmotic gradient, thus drawing water out of the intracellular space [7]. Ultimately, the sperm cells are dehydrated so as to prevent the formation of intracellular ice crystals. Cryoprotectants are generally divided into two classes based on their ability to penetrate the cell membrane, either permeable or impermeable [4]. Permeable agents lower the temperature at which cells are exposed to critical salt concentrations, while impermeable agents work by dehydrating cells allowing them to cool more rapidly before lethal cell damage occurs due to solution effects [4]. Glycerol, a permeable agent, has a long-standing history as the primary cryoprotectant used in freezing stallion semen with the first use as far back as 1950 [5]. Glycerol penetrates the cell membrane by passive diffusion and remains in the membrane and cytoplasm; however, it can lead to cell damage by causing water to move more rapidly leading to dehydration of the cell [3]. Further research has brought attention to glycerol toxicities which include protein denaturation and induction of protein-free membrane blisters [5]. Recent research has led to more common use of amides as cryoprotectants. Amides, like dimethyl-formamide or methyl-formamide, have a lower molecular weight and viscosity when compared with glycerol and may cause less osmotic damage [3,5]. Experiments have shown that amides have increased fertility in "good freezers," although they haven't drastically improved the post-thaw motility of those stallions. They have, however, shown a drastic increase in post-thaw motility of the known "poor freezer" stallions [3,5].

Packaging and Freezing

The extender must be added to the pellet to produce the desired sperm concentration for freezing. Concentrations for cryopreservation range from 100 million sperm/ml to 1.6 billion sperm/ml, with a typical breeding dose falling somewhere between 400 and 800 million total

sperm[8]. Straws should be labeled with information on the stallion's identification, date of processing, breed registration number, and laboratory identification[8]. This can be either hand written or the straws can be ordered with this information pre-printed. Bar codes may also be utilized to scan the straw for the desired information.

Prior to freezing, most extenders require that the straws reach an equilibrium state at 5˚C. The rate at which this should occur will vary depending on the extender, typically around 3-5˚C/min from room temperature [3,9]. Extenders like BotuCrio® (Botupharma, Botucatu, Brazil) require only 20 minutes at 5˚C, while the INRA-96® (IMV, Lisieux, France) suggests 2 hours of cooling [3,9]. The steps taken from here can vary to reach the goal of -196˚C once submerged in liquid nitrogen. The freezing rate must allow an appropriate amount of time for the extracellular water to freeze without allowing ice crystals to form in the intracellular space [7]. One study sites optimal success by decreasing the temperature by 15˚C/min until reaching -80˚C, and then decreasing by 10˚C/min until -120˚C is reached before subsequently plunging the straws into the liquid nitrogen [9]. The vapor box is a commonplace method for freezing, but programmable freezing machines are also available to allow for a more controlled temperature adjustment [3,9]. However, no studies in comparison of the two have shown any significant differences in the overall viability of the sperm [3]. The vapor box is filled with 5 cm of liquid nitrogen and the straws are floated above the nitrogen on a rack. The height above the liquid nitrogen varies with the size of the straws being used, with the larger straws (5 ml) being floated closer to the surface. The 0.5ml straws have been shown to provide better quality on a kinetic level post-thaw than straws of other sizes when the vapor box method is utilized [9]. The 0.5ml straws provide a greater surface area allowing for better control of the freeze rates and are typically floated 3-5 cm above the liquid nitrogen [3]. This increase in surface area allows for a

slower freezing rate, ultimately reducing the cryopreservation injuries associated with more rapid freezing [9].

Thawing and Evaluation

Typically one straw is utilized for a post-thaw evaluation of the sperm on a basis of motility. The temperature at which the straws are thawed also varies with the size of the straw. They can be thawed at low temperatures for a long period of time or at a higher temperature for a shorter time [1]. Typically 0.5-0.25ml straws are thawed at 37˚C for at least 30 seconds [1,6]. Other studies suggest that 0.5ml straws be thawed at 46˚C for 20 seconds or 37˚C for one minute [3,6]. Larger straws like the 4 or 5ml straws are routinely thawed at 50˚C for 45 seconds [6]. Final concentration of the semen in the straws, as well as the motility, progressive motility and morphology should be determined and recorded once the semen is thawed. Generally accepted values are 50% motility and 30% progressively motile sperm [3].

Mare Management

Despite the numerous advantages, breeding on frozen semen requires more diligent management. Acceptable pregnancy rates are much lower on frozen semen, at 40-60% per cycle [3]. Mares considered for frozen semen insemination should be reproductively normal and in good health and body condition [1]. Mares that are older and have repeatedly been unsuccessful breeders should be excluded [1,6]. Tracking the mare through estrus is essential to predict the timing of ovulation and subsequently breeding as close to ovulation as possible. Frozen semen is thought to be able to survive in the reproductive tract for up to 12 hours, thus insemination should take place within a window of 12 hours prior to 6 hours post ovulation if following a synchronized protocol [1,3]. This can be executed by various methods. Single dose protocols are recommended when the quantity of semen is limited. Ovulation is difficult to accurately predict

therefore requiring post-ovulation insemination when a single dose of semen is being used. Mares are given hCG or deslorelin once a 35mm follicle is present in conjunction with uterine edema, followed 12-24 hours later by ultrasound checks every six hours until ovulation is confirmed [1,3,6] The two dose protocols include an ultrasound check and insemination 24 hours post administration of hCG or deslorelen, and a subsequent check at 40 hours to confirm ovulation [1,6]. The second dose of frozen semen will be inseminated once ovulation is confirmed. If ovulation has not occurred at 40 hours, ultrasound checks every six hours are necessary before utilizing the second dose [1,6].

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