Processing stallion semen for on farm use and cooled transport

Dale Kelley[1]

[1]Texas A&M University College Station

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Abstract

Simple dilution of semen is the oldest method to process semen for artificial insemination with either fresh or cooled. This was developed prior to the optimization centrifugation techniques for semen through a combination of trial and error and research. Simple dilution and is a faster method to process semen, is easy to use and works in many circumstances. For on farm semen use a 1:1 (v/v) semen to extender ratio is used. A variety of other techniques have been developed, such as cushion centrifugation, density gradient centrifugation and sperm filtration. Cushion centrifugation is used to concentrate the sperm and allows higher centrifugation speeds than centrifugation alone. This allows for improved recovery rates of sperm. Density gradient centrifugation has been used to select for morphologically normal sperm but suffers from low recovery rates. Processing sperm with this technique can result in improved motility and improved DNA integrity of sperm. Sperm filtration is another method to concentrate sperm without the use of centrifugation; however, this technique has lower recovery rates compared to cushion centrifugation. This review will discuss these techniques in the application of processing equine semen for either fresh or cooled-shipped use.

Processing stallion semen for on farm use and cooled transport

Dale E. Kelley

Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Oklahoma State University, Stillwater, Ok

Summary

Simple dilution of semen is the oldest method to process semen for artificial insemination with either fresh or cooled. This was developed prior to the optimization centrifugation techniques for semen through a combination of trial and error and research. Simple dilution and is a faster method to process semen, is easy to use and works in many circumstances. For on farm semen use a 1:1 (v/v) semen to extender ratio is used. A variety of other techniques have been developed, such as cushion centrifugation, density gradient centrifugation and sperm filtration. Cushion centrifugation is used to concentrate the sperm and allows higher centrifugation speeds than centrifugation alone. This allows for improved recovery rates of sperm. Density gradient centrifugation has been used to select for morphologically normal sperm but suffers from low recovery rates. Processing sperm with this technique can result in improved motility and improved DNA integrity of sperm. Sperm filtration is another method to concentrate sperm without the use of centrifugation; however, this technique has lower recovery rates compared to cushion centrifugation. This review will discuss these techniques in the application of processing equine semen for either fresh or cooled-shipped use.

Introduction

The ideal method for processing semen for use with either fresh or cooled-shipped breeding would maximize the number or sperm per mL, maximize total number of progressively motile sperm, minimize the volume needed to breed a mare, not be deleterious to sperm function or longevity and minimize seminal plasma.

Based on these criteria, cushion centrifugation is currently the best method. Cushion centrifugation maximizes sperm recovery compared to sperm filters(Roach et al., 2016) and centrifugation without cushion. Seminal plasma is removed, which can be deleterious to sperm longevity(Braun et al., 1994; Jasko et al., 1991), allows for the semen to be reconstituted more concentrated than simple dilutions, which reduces the volume of the breeding dose while increasing the number of sperm which inseminated into a mare.

Simple dilution of semen is perhaps the oldest method to extended semen for artificial insemination with either fresh or cooled. This was developed prior to the optimization centrifugation techniques for semen through a combination of trial and error and research. Simple dilution is a faster method to process semen, easy to use and works in many circumstances. A variety of other techniques have been developed, such as density gradient centrifugation, which can be used to remove abnormal sperm and sperm filters which may be useful when a centrifuge is unavailable. This review will discuss these techniques in the application of processing equine semen for either fresh or cooled-shipped use.

Preparation

This review will not go into depth on collection and evaluation of semen; however, it is important to recognized that semen collection and evaluation can greatly impact the quality of the semen after processing. Two important factors in collecting semen which could impact the semen quality are proper washing of the penis (Bowen et al., 1982; Dascanio, 2014) and lubrication of the artificial vaginal. Proper washing reduces smegma in the ejaculate, which, even in small qualities, can be greatly increased if centrifugation is required. Water is recommended for washing, since detergents and disinfectants can increase the risk of pathogenic bacteria colonizing the penis (Bowen et al., 1982). Lubrication of the AV is important, but caution should be taken to minimize the amount of lubricant to reduce contamination of the ejaculate. A non-spermicidal lubrication should be used, but all lubricants are spermicidal to some extent. Clarity brand lubrication has been shown to have the least impact on sperm quality (Serafini et al., 2019). Finally, if more than 2 collection attempts are required with an artificial vagina (AV) to obtain an ejaculate, we recommend a replacing the used AV with a clean, freshly prepared AV to reduce dirt and bacterial contamination.

When evaluating semen, one should look at the color of the ejaculate and a raw sample to determine the presence of sperm or other material such as blood, white blood cells or urine crystals as these can not only impact the accuracy of the evaluation but also the longevity of the sperm. Concentration can be evaluated using a hemocytometer, sphectrophotometer (Equine Densimeter, Spermacue®, etc) or fluorescent based system (nucleocounter®). Each system to measure concentration has its advantages and disadvantages which should be recognized as this can impact accuracy. The hemocytometer allows differentiation of cells (sperm vs other) but is subject to human error either in dilutions, counting sperm or mathematical mistakes. A spectrophotometer-based system works will in situations with little contamination of the ejaculate that is reading on the middle of the standard curve. This system will overestimate sperm numbers if debris, red blood cells, urine or other material is present such as extenders. Additionally, this system is not accurate in cases of low sperm concentration or high sperm concentration. Fluorescent-based systems stain the DNA of the sperm. Thus, non-cellular or red blood cell contamination will not interfere with the accuracy. In cases of pyospermia, this system will overestimate sperm numbers. In situations where the spectrophotometer and fluorescent-based systems are inaccurate, we recommend evaluation with a hemocytometer to try to gain a more accurate sperm concentration. For more in-depth reviews on semen processing and evaluation please see (Jasko, 1992; Love, 2016).

Semen Extender

Several commercial extenders are available for processing semen for both on farm and cooled shipping. It is beyond the scope of this review to discuss the composition and function of extenders and several reviews are available (Aurich, 2008; Brinsko & Varner, 1992; Gibb & Aitken, 2016) .

Breeding dose

The industry standard is to use 500 x 10^6progressively motile sperm for fresh AI and 1 x 10^9progressively

motile sperm for cooled-shipped semen AI (Brinsko, 2006; Douglas-Hamilton et al., 1984; Pickett & Voss, 1975). In reality, some stallions can breed with lower a number of progressively motile sperm and still achieve acceptable pregnancy rates (Brinsko, 2006). Determining the minimum number of progressively motile sperm in a breeding dose for a particular stallion can be done through keeping detailed breeding records.

Insemination volume

An interaction between the total number of sperm inseminated, volume inseminated and sperm concentration has been suggested (Pickett & Shiner, 1994) and sperm concentration may be more important than volume (Katila, 2005). When insemination of mares with a constant total number of sperm, larger volumes reduce pregnancy rates (Jasko, Martin, et al., 1992; Pickett & Shiner, 1994; Rowley et al., 1990), however when the concentration of sperm was consistent (50 x 10^6sperm/ml) there was no difference in pregnancy rate between 30-ml and 120-ml (Bedford & Hinrichs, 1994) of semen. Large insemination volumes (> 50-mL) are not thought to be beneficial as most of the semen will be lost through the mares dilated cervix post insemination (Brinsko et al., 2011; Jones, 1995; Rowley et al., 1990). When considering the inflammation in the mare's uterus post-breeding, the data is confounding. Two studies have found an increase in uterine inflammation with increased sperm concentration(Fiala et al., 2007; Kotilainen et al., 1994). Another study found no effect of sperm concentration on uterine inflammation (Sinnemaa et al., 2005) while a second study found mares infused with 40-mL of extended semen with a concentration of 500 x 10^6 sperm/mL and significantly less uterine inflammation than those infused with 40-mL at a concentration of 50 x 10^6(Nikolakopoulos & Watson, 2000). Thus, it is this author's recommendation to minimize the insemination volume while having enough sperm to produce acceptable pregnancy rates.

Centrifugation for concentrating sperm

Centrifugation can be performed either with or without cushion solution. Cushion is an iodixanol solution which allows the use of higher centrifugation g-forces which reduces sperm damage and improves sperm recovery rates. After semen is analyzed, it is diluted 1:1 using a semen extender. After the semen is diluted, it is transferred to 50-mL conical tubes. If cushion is not being used, the tubes are balanced and centrifuged at 400 x g for 20 minutes. If cushion is being used, after the diluted semen is added to the 50-mL conical tubes, the cushion solution is layered underneath the diluted semen. The amount of cushion solution used can vary on user preference and stallion but is typically between 0.5-mL and 3-mL. The layering of cushion is done by first drawing the cushion solution into syringes and the appropriate volume. Next, a Tom-cat catheter, pipette, or blunted spinal needle can be used to deposit the cushion under the diluted semen. Once this is done the tubes are weighed and balanced and centrifuged at 1000 x g for 20 minutes. It important for the centrifuge to be well balanced as any vibration can disrupt the sperm pellet and reduce the recovery rate of sperm. After centrifugation, the supernatant is removed. This can be done with a pipette or a vacuum aspirator. It is important to remove the supernatant as soon as possible after centrifugation to maximize the sperm recovery rate. Next, the samples are combined and resuspended into one tube and a concentration and volume measured. When using centrifugation to process semen the reconstituted volume can be much lower and sperm concentration much higher than using simple dilution without reducing sperm longevity. Research has found difference in sperm longevity after cushion centrifugation in sperm undergoing cooled-storage at 25 x 10^6 sperm/mL and 250 x 10^6 sperm/mL (Bliss et al., 2012). There is an excellent review on processing semen from subfertile stallions (Varner, 2016) which covers cushioned centrifugation techniques in more detail.

Simple Dilution – On Farm Use

For fresh semen the industry standard is to use 500 x 10^6 progressively motile sperm with semen extender added in a 1:1 (semen: extender) ratio and maintain a volume less than 60-mL. This should then be inseminated into the mare after processing. If there is a short delay from processing to insemination (< 12-hr) then the dose should be stored in a dark location at room temperature until use (Love et al., 2002; Varner et al., 1989). Light and incubator temperatures will adversely affect sperm longevity. If there is a delay longer than 12-hr then the semen should be processed for cooling and held cooled (˜4°C) until use.

Simple Dilution – Cooled-Shipped Semen

The general guidelines for extending semen for cooled-shipping are to have between 5% to 20% seminal plasma (Jasko, Hathaway, et al., 1992), a concentration of 25 x 10^6 to 50 x 10^6 sperm/mL (Varner et al., 1987), a volume less than 50-mL (Loomis, 1992) and 1 x 10^9 progressively motile sperm in the final breeding dose.

Semen extended with 5 to 20% seminal plasma had better motility at 24, 48 and 72 hours of cooled storage (Jasko, Hathaway, et al., 1992). This equates to a dilution ration (semen: extender) from 1:4 to 1:19. Thus, the minimum recommended dilution ratio is 1:4 for cooled-shipped semen. The final extended concentration of sperm should be between 25 to 50 x 10^6 sperm/mL. Dilution of semen to 25 x 10^6 sperm/ml resulted in highest total and progressive motility after 24 h compared to 50 x 10^6 , 100 x 10^6 and no dilution, with the 50 x 10^6 having the second best motility values (Varner et al., 1987). Lastly, The standard recommendation iis to ship [?] 1 x 10^9 progressively motile sperm in the insemination dose (Brinsko, 2006) and minimize the insemination volume (keep it< 50-mL).

There are situations when these guidelines cannot be met such as when a stallion has poorly concentrated semen or poor progressive motility. In these situations, the semen should be concentrated using a technique such as cushion centrifugation, to reduce the total volume while maintaining an acceptable number of progressively motile sperm in the breeding dose. A simplified table (Table 1) was developed to assist in determining the minimum dilution ratio (semen: extender) for cooled-shipped semen based on the raw sperm concentration of semen and the progressive motility cutoff values for centrifugation by sperm concentration in the ejaculate.

Other Semen Processing Techniques

Continuous density gradient centrifugation (also known as single layer colloid) can be a useful technique in some stallions to improve the sperm morphological profile, motility and DNA integrity post processing (Edmond, 2010). One commercially available product is EquiPure^{TM}. It is important to note that this technique does not improve the post processing sperm quality in all stallions, and that a significant number of sperm are lost. Common recovery rates with density gradient centrifugation are from 30% to 40% but can be much lower (Edmond et al., 2012). Thus, it is important to test the effectiveness of this technique on any given stallion prior to shipping semen to mares, by not only looking at the recovery rate but also the morphological profile of sperm after the use of density gradient centrifugation.

Briefly, for this technique semen is extended 1:1 (v/v) with pre-warmed extender. Using either 15-ml or 50-ml conical tubes, the density gradient media (EquiPure^{TM}) is added to the tube first. The volume of EquiPure^{TM} used is typically equal to the amount of extended semen to be added. Next, the extended semen is then added to form a layer on top being careful not to mix the extended semen with the colloid. The tubes are then centrifuged at 400 X g for 20 minutes and afterwards the supernatant removed, and the pellet resuspended. For more details on the procedure the reader is referred to (Bradecamp, 2014a).

Another method to concentrate sperm without the use of centrifugation is through filtration. The recovery rate of sperm using this method was originally reported at 92% and 95%, depending on the size of the pores in the filter (Alvarenga et al., 2010). The sperm recovery rate of the sperm filter is lower compared to cushion centrifugation and differences in motility between the two techniques were negligible, with the authors suggesting this may be a useful technique when centrifugation is unavailable (Roach et al., 2016).

When using the SpermFilter, a petri dish is needed and the semen is extended 2:1 (semen:extender, v/v). The extended semen is added on top of the SpermFilter while it is gently swirled allowing fluid to flow through the filter, collecting sperm on top. Once the sperm has been filtered the membrane is washed with a predetermined volume into a separate petri dish to recover the sperm. For more details please see (Alvarenga & Segabinazzi, 2021).

Packaging and shipping cooled semen

To ensure sperm survival proper packaging is critical. There are several excellent reviews on semen packaging (Aurich, 2008; Bradecamp, 2014b) and thus only certain important points will be highlighted. For packaging, several types of containers can be used such as Whirl-Pak(r) bags, latex/rubber free syringes, conical tubes or screw top tubes. The important point is the storage device does not leak during transportation. Traditionally, it was recommended to remove the air in stored extended semen; however this may not be critical as similar motility parameters and viability have been seen under aerobic and anaerobic conditions (Price et al., 2008). Next, there are many commercial cooled shipping containers. It is recommended to follow the manufacturer's directions for packaging. Finally, make sure to clearly label the packaged semen and include easily identified documentation of the stallion, date and time of collection, sperm concentration, volume of extended semen and motility of the sperm being shipped within the container.

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Table 1. The minimum dilution ratio (semen: extender) for cooled-shipped semen based on the raw sperm concentration of semen and the progressive motility cutoff values for centrifugation by concentration of the ejaculate. If the raw semen sperm concentration is $< 125 \times 10^6$ then centrifugation is needed, for other concentrations the minimum dilution ratio is listed in the adjacent column. In the table are specific concentrations for each range and the corresponding minimum progressive motility needed to avoid having an insemination dose with a volume > 60-ml. **If the progressive motility is less the cutoff value, centrifugation is recommended to avoid having a large insemination volume (> 60-ml).* The progressive motility cutoff values are calculated for a breeding dose with a total number of progressively motile sperm of [?] 1×10^9 and a volume of 60-ml.

Raw Concentration Range (x 10^6 sperm/ml)	**Dilution ratio (Semen: extender/ml)**	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Spec Sper Con cen tra tions of Raw Seme
< 125	**Centrifuge**																			
125 - 250	**1:4**	*125*	*130*	*135*	*140*	*145*	*150*	*155*	*160*	*165*	*170*	*175*	*180*	*185*	*190*	*195*	*200*	*205*	*210*	*215*
250 - 300	**1:5**																		*252*	*258*
300 - 350	**1:6**																			*301*
350 - 400	**1:7**																			
400 - 450	**1:8**																			
450 - 500	**1:9**																			
500-550	**1:10**																			
550-600	**1:11**																			
600 - 650	**1:12**																			
650 - 700	**1:13**																			
700 - 750	**1:14**																			
Progressive Motility																				
Cutoff (%) *	**Progressive Motility**																			
Cutoff (%) *	**67**	**65**	**62**	**60**	**58**	**56**	**54**	**53**	**51**	**49**	**48**	**47**	**45**	**44**	**43**	**42**	**41**	**40**	**39**	**38**

Raw Concentration Range (x 10^6 sperm/ml)	**Dilution ratio (Semen: extender/ml)**	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen	Specific Sperm Concentrations of Raw Semen
Extended Concentration (x10^6 sperm/ml)	**Extended Concentration** **25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**