How to Process a Dilute Ejaculate of Semen for Cooled-Transported Insemination

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To optimize preservation of spermatozoa for cooled-transported insemination, dilute ejaculates of semen containing <100 million spermatozoa/ml are generally centrifuged in order to concentrate the spermatozoa and remove some of the seminal plasma. Centrifugation is performed by mixing raw semen 1:1 with extender and dispensing the extended semen in 40-ml aliquots and spinning for 10 to 12 minutes at a centrifugal force of 300 to 400 times gravity. This results in the recovery of approximately 75% of the spermatozoa in each tube. After centrifugation, 30 ml of the supernatant is removed and 20 ml fresh extender is added to each tube and the pellet is resuspended, which empirically produces a final concentration of spermatozoa approximately one-half the initial concentration (in the raw semen) in a mixture containing 17% seminal plasma. Author’s address: Northwest Equine Reproduction Laboratory, Department of Animal and Veterinary Science and Center for Reproductive Biology, University of Idaho, Box 442201, Moscow, ID 83844; e-mail: dirkv@uidaho.edu. © 2008 AAEP.

1. Introduction

With the exception of the Jockey Club, all major equine breed registry associations allow the use of cooled-transported semen for breeding, and its use in the equine breeding industry is now a standard practice. Routine procedures for processing an ejaculate of semen to prepare it for cooled-transported insemination are well described and generally involve mixing the semen with a suitable extender (≤3:1 ratio of extender to semen) to produce a final concentration of 25–50 million spermatozoa/ml. The volume of extended semen needed to provide at least one billion progressively motile spermatozoa (before cooling) in each insemination dose is determined, and each dose is packaged and placed into a shipping container for passive cooling to ~5°C during transportation to its final destination for insemination. Although routine semen processing procedures are suitable for most ejaculates, a dilute ejaculate with a concentration of spermatozoa <100 million/ml generally requires more extensive processing. Specifically, dilute semen should be centrifuged to remove seminal plasma and concentrate the spermatozoa; otherwise, the required minimum 3:1 dilution ratio would result in a final concentration of spermatozoa below the lower threshold of 25 million/ml that is necessary for retention of optimum viability of the spermatozoa during the cooling/storage period. Although intuitively it would seem a lower dilution ratio (e.g., 1:1 or 2:1 rather than 3:1) could be used to keep the final concentration of spermatozoa in the desired range of 25–50 million/ml, doing so would result in insufficient dilution of the seminal plasma, because the seminal plasma should be diluted to 25% or less of its original concentration to maximize preservation of spermatozoal viability during cooling/storage.
Processing a dilute ejaculate of semen involves mixing the semen with extender and centrifuging the extended semen to concentrate the spermatozoa in a soft pellet at the bottom of a centrifuge tube. After centrifugation, some of the supernatant (a 1:1 mixture of seminal plasma and extender) is aspirated and discarded (removing seminal plasma), fresh extender is added, and the spermatozoa in the pellet are resuspended. The objective is to produce a final concentration of spermatozoa between 25 and 50 million/ml in a mixture of extender and seminal plasma containing 5–20% seminal plasma.1 Centrifugation can be performed with or without the use of a centrifugation “cushion,” a dense liquid medium placed below the extended semen that prevents excessive compaction of the spermatozoa at the bottom of the tube. The use of a cushion allows more vigorous centrifugation (i.e., increased centrifugal force and/or time), which helps maximize recovery of spermatozoa without excessive damage. This paper describes how to centrifuge semen without a cushion, because it is unlikely the specialized cushion solution will be available when initially faced with the need to process a dilute ejaculate of semen. If dilute semen is routinely encountered, a commercially available cushion solution can be obtained for subsequent use; additional information about the use of a centrifugation cushion is provided in the discussion section.

2. Materials and Methods

Equipment and Supplies
In addition to the materials necessary for routine procedures involved with the collection, evaluation, and processing of equine semen, the following equipment and supplies are needed:

1. Table-top centrifuge with a swinging-bucket rotor that accommodates 50-ml centrifuge tubes and can generate a relative centrifugal force of 300-1000 times gravity (g-force; Fig. 1)
2. Disposable sterile plastic conical bottom 50-ml centrifuge tubes
3. Disposable sterile “serologic” pipettes (5 and 10 ml) and a manual pipettor (e.g., bulb or thumb-wheel style) for aspirating supernatant fluid

Because most centrifuges allow adjustment of the revolutions per minute (RPM) but do not provide a means of directly setting the g-force, it is important to determine the RPM needed to generate a specific g-force on an individual centrifuge. To calculate g-force, the rotating radius of the centrifuge rotor is determined by measuring the distance from the center point of the rotor to the bottom of a bucket in its “swinging” position (Fig. 2); plotting the length of the rotating radius against RPM on a relative cen-
A centrifugal force nomograph chart gives the g-force at any given RPM (Fig. 3). The RPM can be set to provide the desired g-force during centrifugation. Once the RPM/g-force is determined, the duration (in minutes) of centrifugation can be set to provide the desired combination of time and centrifugal force.

Procedure (With a Hypothetical Example)

1. Immediately after collection, the entire ejaculate is mixed with an equal volume (1:1) of warm (37°C) extender, decreasing the concentration of spermatozoa by one half.

Example: An ejaculate is obtained that has a gel-free volume of 80 ml and a concentration of 74 million spermatozoa/ml (5.92 billion total spermatozoa); mixing the entire ejaculate with an equal volume of extender results in an extended volume of 160 ml with a concentration of 37 million spermatozoa/ml.
2. The entire volume of extended semen is dispensed in 40-ml aliquots into sterile 50-ml centrifuge tubes. In most instances, after dispensing the extended semen in 40-ml aliquots, there will be one partial aliquot containing <40 ml; in that case, place the partial aliquot in a tube and centrifuge with the other tubes as outlined below. Subsequent handling of a partial tube is covered in step 10.

- Example: The 160-ml volume of extended semen is dispensed into four equal 40-ml aliquots in the centrifuge tubes. There is no partial aliquot in this example.

3. Determine the total number of spermatozoa in each tube by multiplying the volume (40 ml) times the concentration of spermatozoa in the extended semen.

- Example: 40 ml/tube \times 37 million/ml = 1480 million spermatozoa/tube (1.48 billion/tube).

4. Place the centrifuge tubes into the buckets on the centrifuge rotor ensuring that each tube is adequately balanced and run the centrifuge at room temperature for 10–12 min at 300–400 g; the goal is to obtain a “soft” sperm pellet (the optimum combination of time and g-force will vary among stallions).

5. Under these centrifugation conditions, it is empirically estimated that ~75% of the spermatozoa will be recovered in the pellet; based on that assumption, determine the number of spermatozoa in the pellet by multiplying the total number of spermatozoa in the tube (from step 3 above) by 0.75.

- Example: 1480 million spermatozoa/tube \times 0.75 = 1110 million spermatozoa in the pellet in each tube.

6. Using a sterile serological pipette, aspirate (and discard) 30 ml of the supernatant from each tube, leaving 10 ml of supernatant with the pellet. Because the supernatant is a 1:1 mixture of extender and seminal plasma, the tube now contains 5.0 ml of extender and 5.0 ml seminal plasma.

7. Add 20 ml of fresh extender (at room temperature) to each tube to bring the total volume to 30 ml/tube and resuspend the spermatozoa in the pellet by gently mixing the contents of the tube. This produces a final volume in each tube that is 75% of the original volume (30 ml/40 ml), which, coupled with the 75% recovery rate of spermatozoa in the pellet, results in a concentration of spermatozoa approximately the same as in the original extended semen before centrifugation. If desired, the actual concentration of spermatozoa can be determined using a hemacytometer. The addition of 20 ml fresh extender to reach the final volume of 30 ml results in a concentration of seminal plasma of ~17% in the final sample (5 ml seminal plasma in 30 ml; see step 6).

- Example: Approximately 1110 million spermatozoa were recovered in the pellet; therefore, resuspension of the pellet in 30 ml produces a final concentration of spermatozoa of 37 million/ml (1110 million spermatozoa/30 ml) with a final concentration of seminal plasma of 17%.

8. Evaluate the post-centrifugation motility of the spermatozoa and determine the number of progressively motile spermatozoa in each tube by multiplying the total number of spermatozoa in the tube in the pellet after resuspension of the pellet (from step 5) by the percentage progressively motile spermatozoa.

- Example: Post-centrifugation progressive motility is 70%; therefore, 1110 million spermatozoa/tube \times 0.70 = 777 million progressively motile spermatozoa/tube (in 30 ml).

9. Pool the extended semen and prepare individual insemination doses each containing at least one billion progressively motile spermatozoa.

- Example: There are four tubes, each containing 777 million progressively motile spermatozoa in a volume of 30 ml, so the cumulative volume of 120 ml contains a total of 3108 million (3.1 billion) total progressively motile spermatozoa; therefore, a maximum of three doses, each containing just over 1 billion progressively motile spermatozoa in 40 ml, are available for insemination from this ejaculate after processing.

10. The procedure for processing a partial tube that was initially filled with <40 ml of extended semen will be slightly different than what is described above. At step 6, instead of aspirating 30 ml of supernatant, remove proportionately less supernatant based on the amount of extended semen originally placed into the tube before centrifugation.

- Example: If a tube was originally filled with 24 ml of extended semen, remove 18 ml of supernatant (leaving 6 ml in the tube with the pellet); 24 ml is 60% of 40 ml, so 60% of 30 ml is 18 ml.

- Then at step 7, instead of adding 20 ml of fresh extender, add proportionately less based on
the amount of extended semen originally placed into the tube before centrifugation.

- Example: As above, if a tube was initially filled with 24 ml of extended semen, add 12 ml fresh extender (after removing 18 ml); 24 ml is 60% of 40 ml, so 60% of 20 ml is 12 ml. This results in a final extended volume of 18 ml (6 ml left with pellet plus 12 ml fresh extender) in this tube with the same concentration of spermatozoa as the tubes that had been originally filled with 40 ml. After resuspending the pellet in the partial tube, its contents are pooled with the extended semen from the other tubes as described in step 9.

3. Discussion

This procedure for processing a dilute ejaculate of semen is particularly well suited for ejaculates that have an initial concentration of spermatozoa between 50 and 100 million/ml, because it will empirically result in a concentration of spermatozoa approximately one half the original concentration (i.e., 25–50 million/ml) in a solution containing 17% seminal plasma, both of which are within the optimal range for cooling. If the initial concentration of spermatozoa is <50 million/ml, steps 1 through 5 of the protocol are the same; however, beginning at step 6, it is necessary to calculate exactly how much supernatant to leave in each tube and the volume of fresh extender to add to produce a final concentration of spermatozoa between 25 and 50 million/ml with a concentration of seminal plasma of 5–20%. By doing these calculations beginning at step 6, it is possible to set the final concentration of both spermatozoa and seminal plasma at a specific level within their optimum ranges and remove supernatant and add fresh extender as needed to achieve the desired result.

As noted previously, centrifugation can be performed with or without the use of a centrifugation “cushion,” a dense liquid medium placed at the bottom of the centrifuge tube that prevents excessive compaction of the spermatozoa. When centrifuging without a cushion, as described in the protocol outlined above, ~75% of the spermatozoa are recovered in the pellet. With the use of a centrifugation cushion, recovery rates approaching 100% can be achieved, because the cushion allows more vigorous centrifugation of the sample (e.g., 400-1000g for 20–25 min). A cushion is prepared by carefully layering 3.0–4.0 ml of cushion medium beneath the extended semen at the bottom of the centrifuge tube before centrifugation. After centrifugation, the spermatozoa are suspended in a pellet at the interface of the cushion material and extender. Before resuspending the spermatozoa in the pellet, the cushion material should be removed by carefully passing a sterile pipette through the supernatant and pellet into the cushion material and aspirating it. Because of the higher recovery rate of spermatozoa when using a cushion, the amount of supernatant removed and volume of fresh extender added must be adjusted accordingly to produce a final concentration of spermatozoa of 25–50 million/ml and a seminal plasma concentration of 5–20%. If dilute semen is routinely encountered, and/or the total number of spermatozoa in an ejaculate are extremely limited, the use of a centrifugation cushion may be particularly beneficial to optimize recovery of the spermatozoa. In addition to the use of a centrifugation cushion when processing dilute semen for cooled-transported insemination, centrifugation with a cushion is being used increasingly when processing semen for freezing to maximize the number of insemination doses that can be produced from each ejaculate.

References and Footnotes


aCushion Fluid. Minitube of America, Verona, WI 53593.
bEqcellsire. IMV USA, Maple Grove, MN 55369.
cOptiPrep Universal Centrifugation Cushion, Exodus Breeders, York, PA 17406.