# Optimization of a protocol for cryopreservation of electroejaculated beef bull semen under ambulatory conditions

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**Abstract**

The overall goal was to optimize and simplify a cryopreservation protocol under ambulatory conditions. It was hypothesized that removing seminal plasma by centrifugation would improve post-thaw semen quality, and that increasing equilibration to 24 h and freezing in liquid nitrogen vapor within the neck of the tank would not affect post-thaw quality of electroejaculated bull semen. One ejaculate was collected from nine beef bulls using electroejaculation, and was divided into four aliquots: NC5: non-centrifuged, equilibrated for 5 h; NC24: non-centrifuged, equilibrated for 24 h; CE5: centrifuged, equilibrated for 5 h; CE24: centrifuged, equilibrated for 24 h. Straws were held horizontally on a rack 3cm above liquid nitrogen for 10min before plunging in liquid nitrogen. In addition, some straws equilibrated for 5h were held vertically 2cm over liquid nitrogen within the neck of the tank (NCneck, CEneck). Post-thaw sperm motility (CASA), membrane integrity (SYBR14/PI), acrosome integrity (FITC-PNA) and apoptosis (ANV) were evaluated and compared using ANOVA. Sperm motility, membrane integrity or apoptosis did not differ among treatments. However, centrifuged semen equilibrated for 24h had significantly lower DSL, VSL, LIN, STR and WOB than non-centrifuged semen equilibrated for 5h. Centrifugation increased the percentage of acrosome-damaged spermatozoa (NC5 5.8±3, CE5 42.2±10.7, NC24 4.8±2.5, CE24 32.5±8.9, NCneck 17.9±7.2, CEneck 42.3±12.5%; P=0.002). Extending the equilibration time had no effect on the parameters evaluated in non-centrifuged semen. Removal of seminal plasma by centrifugation is not recommended since it had a negative effect on acrosome integrity.

**Keywords:** Bull, cryopreservation, centrifugation, equilibration, liquid nitrogen vapor

## Introduction

Use of frozen semen has become the standard for artificial insemination in cattle. Current management of bulls for semen freezing involves housing bulls at specialized centers. The bulls are trained to ejaculate into an artificial vagina, and semen is collected and frozen on a regular basis until the desired number of insemination doses is obtained. Such practice involves a high cost to bull owners related to animal transportation, quarantine and housing, in addition to the cost of semen freezing itself. Small producers, especially of rare breeds, often want to cryopreserve semen from bulls that have genetic value for their programs, but cannot afford the cost of semen freezing at a specialized facility. The optimization of a method to collect and freeze semen at the farm would reduce the cost of custom-freezing bull semen and favor a more widespread use of this technology.

Most of the reports in the literature describe and address cryopreservation of dairy bull semen in semen freezing centers, collected with an artificial vagina, and processed under controlled laboratory conditions. Such bulls are intensively selected based on their production of excellent quality semen with good freezing ability.[1] Some of the protocols used and reported may not be suitable for client-owned beef bulls selected for the genetic value to the producer's breeding program and not for their freezing ability. Cryopreservation under ambulatory conditions requires making adjustments to protocols to overcome specific challenges. These challenges are presented by the working conditions in ambulatory practice and critically involve the lack of facilities and personnel trained for semen collection with an artificial vagina, long working hours, time constraints due to other routine duties, uncontrolled environment with exposure to unfavorable ambient conditions, and limited availability of equipment like automated semen freezers. Therefore, the overall goal of this study was to advance the art of on-farm semen freezing by the

optimization and simplification of a method to freeze electroejaculated beef bull semen under ambulatory conditions.

Collecting and processing semen for cryopreservation on-farm requires the use of electroejaculation, which yields samples with variable concentrations of seminal plasma, and which can be contaminated with urine. While the removal of seminal plasma is not routine practice in the cattle industry, seminal plasma is routinely removed before freezing equine and canine semen. It has been demonstrated that this practice improves survival and membrane stability of spermatozoa during cryopreservation.[2] Therefore, removal of seminal plasma and other contaminating fluids may improve the post-thaw quality of frozen bovine semen obtained by electroejaculation. However, reports provide conflicting data of the costs and benefits of centrifugation of bovine semen. In some studies, sperm velocity, mitochondrial potential, oxidative stress and DNA integrity were improved by centrifugation and removal of seminal plasma prior to cryopreservation.[3,4] In contrast, sperm motility and membrane integrity did not change, and acrosome integrity and *in vitro* fertility decreased with centrifugation in one of these studies.[4] Because of the variation and discrepancy in findings, the effect of centrifugation on post-thaw bovine semen quality is still not clear and needs to be further evaluated. Furthermore, no studies have evaluated the effect of centrifugation in *Bos taurus* beef bulls collected with electroejaculation. It is unknown if breed differences may exist in seminal plasma composition that may result in differential effects on beef bull semen quality when it is removed.

Optimization of time management is another critical factor for a busy ambulatory practice. Most current protocols involve an equilibration time of at least 2h,[5] time during which a clinician is prevented from providing other services while waiting at the farm. The ability to equilibrate semen overnight would allow transport of semen to the laboratory during equilibration. There the freezing process could be completed under controlled conditions on the following day. This would reduce the number of work hours required for freezing of semen, or allow practitioners to peruse other activities while the semen equilibrates. Previous studies have demonstrated that equilibration of semen overnight may actually be beneficial for post-thaw quality of semen collected with an artificial vagina in a controlled environment from dairy bulls.[1,6] However, transient exposure of semen to varying environmental and handling conditions at the farm could result in sublethal damage that may affect sperm survival during prolonged times at refrigeration temperature. Furthermore, the interaction between removal of seminal plasma by centrifugation and equilibration time has not been previously evaluated in cattle.

Finally, if the semen freezing process is completed on the farm, an automated semen freezer is rarely available and a manual method is most often required. The straws are loaded under refrigeration conditions and frozen in liquid nitrogen vapor phase. This is performed by holding straws horizontally on a rack, 3 to 6cm above liquid nitrogen.[5,7] Therefore, a container capable of safely holding liquid nitrogen and fitting the rack needs to be available. After rack freezing, the straws are plunged in the liquid nitrogen and placed into goblets, which are transferred to the tank for storage. The freezing process could be simplified and the amount of materials needed could be minimized if the straws could be held vertically in the liquid nitrogen vapor within the neck of the storage tank.

The objectives of this study were to evaluate the effect of removal of seminal plasma by centrifugation, prolongation of the equilibration time and freezing in the liquid nitrogen vapor within the neck of the tank on post-thaw semen quality. It was hypothesized that removing seminal plasma by centrifugation would improve post-thaw semen quality, and that increasing the equilibration time to 24 h and freezing in the liquid nitrogen vapor within the neck of the tank would not affect post-thaw quality of electroejaculated beef bull semen.

## Materials and methods

### Experimental animals

Nine 17-month old Angus bulls were used in the study. No attempt was made to select bulls based on freezing ability to replicate commercial conditions of the local industry. Initial total and progressive sperm motility was 65.2 ± 4.3% and 62.7 ± 4.4%, respectively (mean ± SEM). Mean percentage of morphologically normal spermatozoa was 71.4 ± 3.6%. Three bulls had less than 70%

normal spermatozoa (46, 64 and 65% normal spermatozoa). The main finding in these three bulls was presence of distal droplets. While spermatozoa with distal droplets were not counted as morphologically normal here, presence of distal droplets does not affect fertility in bulls and is not considered a morphological defect by many clinicians. In addition, these bulls had sperm post-thaw motility comparable with the other bulls. Therefore, they were included in the study. Mean sperm concentration, volume and total sperm count was 492.2 ± 109.9 x $10^6$ spermatozoa/ml, 4.5 ± 0.3 ml and 2.1 ± 0.5 x $10^9$ spermatozoa, respectively. Scrotal circumference ranged between 34.5 and 38 cm. No abnormalities were noted in the prepuce, penis, scrotum or accessory sex glands of any of the animals. Bulls were group-housed in one pasture at the University of Georgia's research farm, and were fed water, hay and mineral salt *ad libitum*. The nine bulls were included in the first experiment. Three of the bulls were then turned out with the university's cow herd for breeding and were unavailable for the second study. Therefore, six bulls were included in the second experiment. The study was performed following guidelines from the Institutional Animal Care and Use Committee.

Experiment 1: Effect of centrifugation, equilibration time and vapor freezing method on post-thaw semen quality

A cleanout semen collection was performed one week prior to the study. On the day of the study, one ejaculate was obtained from each of nine bulls. The cloudy sperm-rich fraction was collected using the automatic setting on an electroejaculator (Pulsator IV, Lane Manufacturing Inc., Denver, CO) during penis protrusion or erection. Sperm concentration was evaluated using a densimeter. Immediately after collection, 200µl of an antibiotic cocktail (CSS Antibiotic Mix, IMV Technologies, Maple Grove, MN) was added per ml of raw semen. Each 200µl of antibiotic cocktail contained 100µg of tylosin, 500µg of gentamycin and 300/600µg of linco-spectin. Semen was then placed in a water bath at 34°C for 5min. Pre-warmed (34°C; OptiXcell®, IMV Technologies) was added to dilute the semen to make a final concentration of 50 x $10^6$ spermatozoa/ml. Each ejaculate was then divided into four aliquots (Table): NC5: non-centrifuged, equilibrated for 5h; NC24: non-centrifuged, equilibrated for 24h; CE5: centrifuged, equilibrated for 5h; CE24: centrifuged, equilibrated for 24 h. Non-centrifuged aliquots (NC5 and NC24) were refrigerated immediately after dilution. Centrifuged aliquots (CE5 and CE24) were centrifuged at 800 x g for 10min, the supernatant was removed and the pellet was resuspended to 50 x $10^6$ spermatozoa/ml in OptiXcell®. Semen collection, dilution and centrifugation were performed bull-side at the farm. The extended semen was initially placed within the truck's refrigerator at the farm. Transportation time from the farm to the laboratory was approximately 30min. Upon arrival at the laboratory, semen was moved to the laboratory's refrigerator within a pre-cooled closed-cell extruded polystyrene box to complete equilibration at 5°C for 5h (NC5 and CE5) or 24h (NC24 and CE24). Cooling rate was not calculated in this study, but bovine spermatozoa tolerate variable cooling rates between 50 and 100°C/min.[5] After equilibration, pre-cooled 0.5ml straws were loaded. Next, the straws were placed horizontally on a rack 3cm above liquid nitrogen for 10min within a closed-cell extruded polystyrene box. The straws were then plunged in liquid nitrogen, loaded into 13-mm goblets submerged in the liquid nitrogen, and transferred to the storage tank. In addition, half of the straws equilibrated for 5h, both non-centrifuged and centrifuged (NCneck and CEneck), were exposed to liquid nitrogen vapor within the neck of the storage tank (Table). The straws were loaded into pre-cooled 13-mm goblets within the refrigerator. Only the bottom goblet of each cane was filled with straws. The canes were then held vertically within the neck of the tank so that the bottom of the goblet was 2cm above the liquid nitrogen. The distance from liquid nitrogen was modified to ensure all semen and extender mixture within the straw theoretically reached a temperature of -40°C at the warmest point before plunging into liquid nitrogen. This is the temperature at which 95% of water in the extended semen is frozen.[7] While temperature was not measured in this study, it was expected to be -160°C at the bottom of the straw (2 cm above liquid nitrogen) and -40°C at the top of the straw (15 cm above liquid nitrogen).[7] After 10min, the canes were lowered to rapidly submerge the straws into the liquid nitrogen. All straws were stored at -196°C until evaluation. Two weeks after freezing, one straw from each aliquot was thawed in a water

bath at 38°C for 30s, and sperm motility, membrane integrity, acrosome integrity and apoptosis were evaluated (see Evaluation of sperm parameters). Statistical analysis was performed using Statistical Analysis Software (SAS, SAS Institute, Cary, NC). Normally distributed data were expressed as mean ± SEM. Mean motion parameters, percentage of membrane intact, acrosome intact and apoptotic spermatozoa were compared among the six treatments (NC5, NCneck, NC24, CE5, CEneck and CE24) using ANOVA, including the fixed effect of treatment, and the interaction between centrifugation and equilibration time. Paired comparisons were made using a Tukey's test. Differences were considered significant if $P < 0.05$.

Table. Summary of treatments and conditions

| Treatment | NC5 | NCneck | NC24 | CE5 | CEneck | CE24 |
|---|---|---|---|---|---|---|
| Centrifugation | no | no | no | yes | yes | yes |
| Equilibration time (h) | 5 | 5 | 24 | 5 | 5 | 24 |
| Vapor freezing | horizontal | vertical | horizontal | horizontal | vertical | horizontal |

Experiment 2: Effect of centrifugation on sperm motion and acrosome integrity in fresh semen

A negative effect of centrifugation on acrosome integrity of frozen-thawed semen was noted in the first experiment. Experiment 2 was performed to determine if this effect was caused by mechanical damage during centrifugation, or by cryodamage resulting from removal of seminal plasma. One week after the first experiment, another ejaculate was collected from each of six bulls using electroejaculation. Pre-warmed (34 °C) OptiXcell® was added to make a final concentration of 50 x $10^6$ sperm/ml. Each ejaculate was then divided into two aliquots. One aliquot served as the non-centrifuged (NC) control treatment while the other aliquot was centrifuged at 800 x g for 10min (CE). The supernatant was removed and the pellet was resuspended to a final concentration of 50 x $10^6$ sperm/ml in OptiXcell®. Evaluation of sperm motility, membrane integrity and acrosome integrity was then performed in fresh semen (see Evaluation of sperm parameters). Semen was not frozen in this experiment. Mean motion parameters, percentage of membrane intact and acrosome intact spermatozoa were compared between centrifuged and non-centrifuged aliquots using a T test.

Evaluation of sperm parameters

Phase contrast microscopy was used for assessment of sperm morphology immediately after semen collection. Spermatozoa were diluted 1:10 in formalin buffered solution (Animal Reproduction Systems, Chino, CA). A wet mount was prepared and 100 spermatozoa were classified based on their morphological characteristics under oil immersion at X100 magnification. Sperm motility was evaluated with a computer assisted semen analyzer (CASA; SpermVision Professional, Minitube of America, Verona, WI). The settings of the instrument were: field depth of view 20µm, pixel to µm ratio 130 to 100, cell area 18 to 80µm, frames acquired 30, frame rate 60Hz, AOC cut off static cells 5 and DSL cut off 4.5µm/s (progressive motility). Mean percentage of total (TMOT) and progressively (PMOT) motile spermatozoa was assessed from all cells present in seven fields with a X 20 phase-contrast objective. Other motion parameters analyzed were: average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN), wobble (WOB), curvilinear distance (DCL), straight line distance (DSL), and distance of average path (DAP).

For evaluation of acrosome and plasma membrane integrity, 1µL of a 1µg/mL solution of fluorescein isothiocyanate-labeled peanut agglutinin (FITC-PNA; Cat. No. F-2301-1, EY Laboratories, San Mateo, CA) and 1µL of a 6µM solution of propidium iodide (PI; Live/Dead Kit, Molecular Probes) were added to a 1mL suspension containing 5 x $10^6$ spermatozoa. Semen was incubated at 38°C in 5% $CO_2$ in air for 10min. The percentage of membrane-intact (PI negative) and live acrosome-damaged (PI negative and PNA positive) spermatozoa was evaluated by flow cytometry.

Sperm apoptosis was evaluated using FITC-labelled Annexin V stain (ANV, Cat. No. A13199, Molecular Probes, Eugene, OR). Spermatozoa were washed once by centrifugation at 600 x g for 5min and were resuspended to 4 x $10^5$ /ml in binding buffer (0.1 M HEPES, 1.4 M sodium chloride, 25mM calcium chloride in distilled water, pH 7.4). Then, 10µl of FITC-Annexin V was added to 1ml of sperm suspension, and semen was incubated for 15min at room temperature in the dark. Semen was washed by centrifugation once to remove unbound stain, and was resuspended to 0.5mL in binding buffer. Then 0.5µL of a 6µM solution of PI was added. Cells were evaluated by flow cytometry and were classified as membrane-intact (ANV negative/PI negative), early apoptotic (ANV positive/PI negative), late apoptotic (ANV positive/PI positive), or necrotic (ANV negative/PI positive).

A fluorescence flow cytometry analyzer (Accuri™ C6 Generation 1, BD Biosciences, San Jose, CA) was used in this study. From each sample, 10,000 events in the forward and 90º light scatter population representing whole sperm were analyzed using 0.2-µm filtered 18 mega-ohm ultra pure water as the sheath fluid. A gate containing spermatozoa was selected based on dot plot distribution of forward (size) versus side scatter (complexity parameter) to eliminate debris and epithelial cells from the analysis. The FITC (green) and PI (red) signals were detected using a 5mWatt blue argon laser (488 nm) and emission filters (535 ± 30 nm for FITC and 585 ± 30 nm for PI). Baseline background fluorescence signal was initially evaluated in unstained samples. Compensation for FITC emission into the PI detector or vice versa was done by adjusting digital compensation to remove the green signal from the red detector and the red signal from the green detector with samples containing only FITC or PI. The areas of single and dual staining were established by setting quadrants on spermatozoa labeled only with PI or FITC. Fluorescence emission data were collected using a 7.2 log logarithmic digital signal collection system for green fluorescence (FITC) in the FL1 detector and red fluorescence (PI) in the FL3 detector. The control quadrant (lower left) was marked on unstained samples with < 1% of cells registering as positive in the upper left, upper right and lower right quadrants.

**Results**

Experiment 1: Effect of centrifugation, equilibration time and vapor freezing method on post-thaw semen quality

The percentage of total and progressively motile spermatozoa was not affected by treatment. However, there was an effect of treatment on DSL (P=0.038), VSL (P=0.045), LIN (P=0.008), STR (P=0.007) and WOB (P=0.034; Fig. 1). There was also an interaction between centrifugation and equilibration time on LIN (P=0.007), STR (P=0.005) and WOB (P=0.028). Centrifuged semen equilibrated for 24 h had lower DSL, VSL, LIN, STR and WOB than non-centrifuged semen equilibrated for 5h frozen traditionally or within the neck of the tank (Fig. 1). No other motion parameters differed.

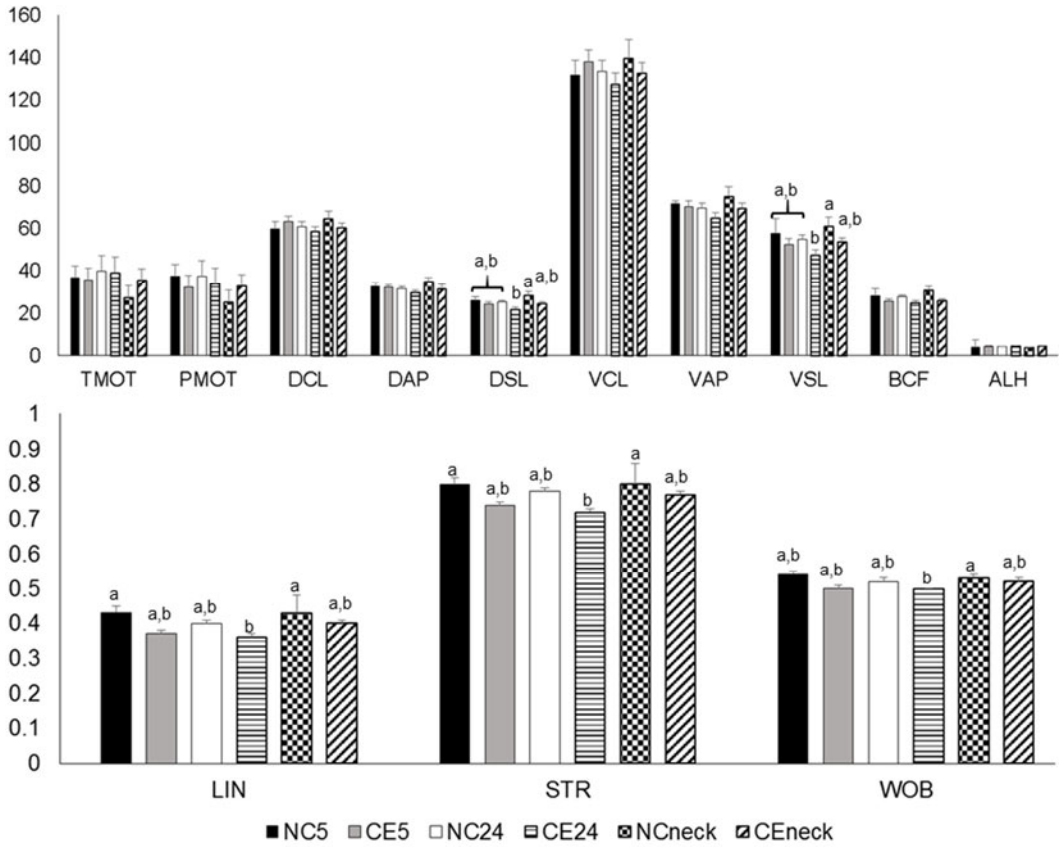


Figure 1. Sperm motion parameters in centrifuged (CE) and non-centrifuged (NC) frozen-thawed bovine semen equilibrated for 5 h(5) or 24 h(24), and exposed to liquid nitrogen vapor within the neck of the tank (neck). [a,b]P<0.05. TMOT=total motility (%), PMOT=progressive motility (%), DCL=curvilinear distance (μm), DAP=distance of average path (μm), DSL=straight line distance (μm), VCL=curvilinear velocity (μm/sec), VAP=average path velocity (μm/sec), VSL=straight line velocity ((μm/sec), LIN=linearity, STR=straightness, WOB=wobble, BCF= beat cross frequency (hertz), ALH= amplitude of lateral head displacement (μm).

The percentage of membrane-intact, necrotic, early apoptotic and late apoptotic cells did not differ significantly with treatment (Fig. 2). The percentage of live spermatozoa with damaged acrosomes was higher in centrifuged than non-centrifuged samples (P=0.002; Fig. 2). When semen was frozen at the neck of the tank, acrosomal damage did not differ with centrifugation but was higher than in non-centrifuged samples frozen traditionally. There was also an interaction between centrifugation and equilibration time on late apoptotic cells (P=0.014) and live acrosome damaged cells (P=0.001).

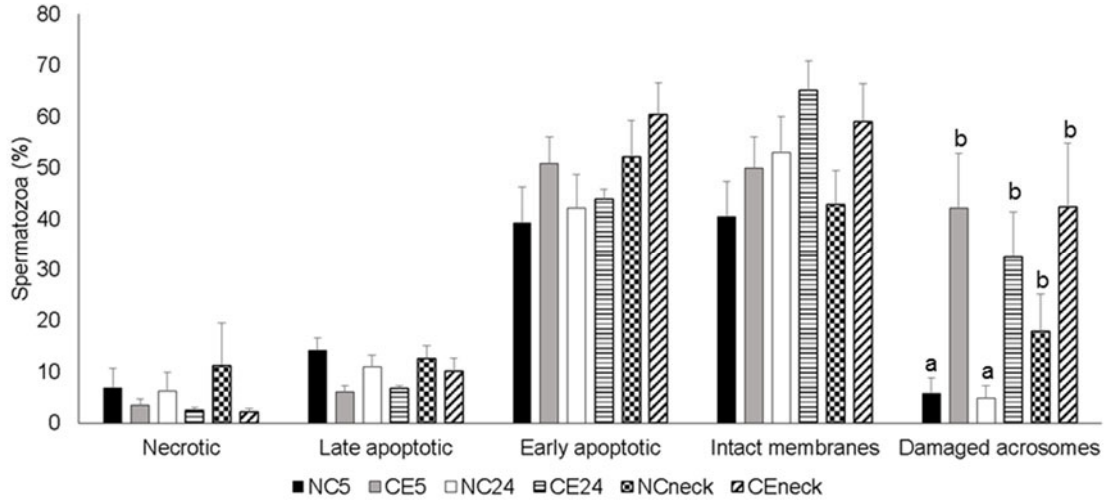


Figure 2. Percentage of necrotic, apoptotic, membrane-intact and live acrosome-damaged spermatozoa in centrifuged (CE) and non-centrifuged (NC) frozen-thawed bovine semen equilibrated for 5 h (5) or 24 h (24), and exposed to liquid nitrogen vapor within the neck of the tank (neck). $^{a,b}P=0.002$.

Experiment 2: Effect of centrifugation on sperm motion and acrosome integrity in fresh semen

Sperm motion parameters, plasma membrane integrity and acrosome integrity did not differ between centrifuged and non-centrifuged fresh semen (P>0.05; Fig. 3).

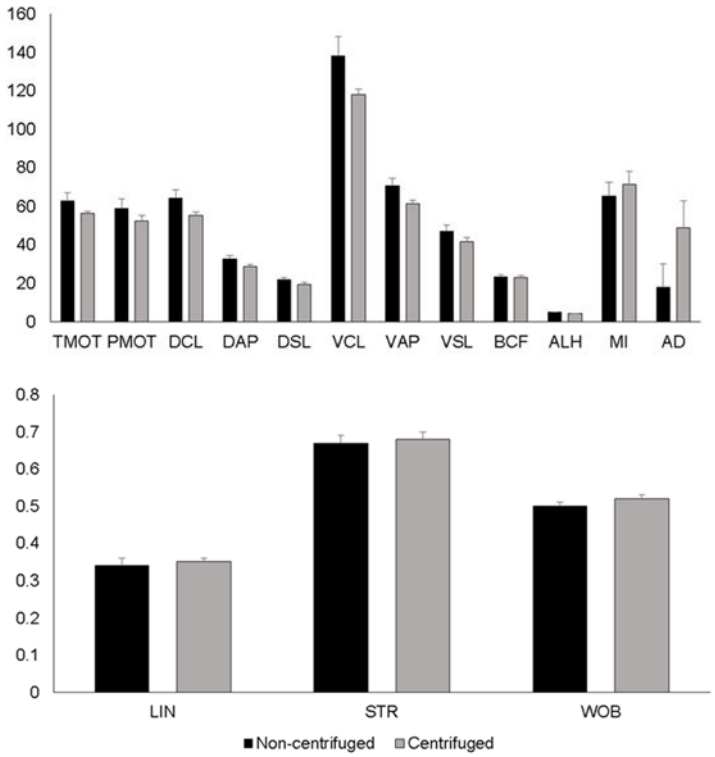


Figure 3. Sperm motion parameters, plasma membrane integrity and acrosome integrity in centrifuged (CE) and non-centrifuged (NC) fresh bovine semen. TMOT=total motility (%), PMOT=progressive motility (%), DCL=curvilinear distance (μm), DAP=distance of average path (μm), DSL=straight line distance (μm), VCL=curvilinear velocity (μm/sec), VAP=average path velocity (μm/sec), VSL=straight line velocity ((μm/sec), LIN=linearity, STR=straightness, WOB=wobble, BCF= beat cross frequency (hertz), ALH= amplitude of lateral head displacement (μm), MI=membrane integrity (% spermatozoa), AD=damaged acrosomes (% spermatozoa).

**Discussion**

This study evaluated ways to optimize and simplify the protocol for cryopreservation of electroejaculated bovine semen under ambulatory conditions. Centrifugation was performed to remove seminal plasma and other contaminating fluids from electroejaculated samples, to minimize the interaction of the sperm plasma membrane with seminal plasma proteins and these contaminating fluids. However, centrifugation had no beneficial effect on sperm motility, membrane integrity or apoptosis. Furthermore, there was an interaction between centrifugation and equilibration time that resulted in a negative effect of removing seminal plasma on some motion parameters when semen was equilibrated for 24h.

A negative effect of bovine seminal plasma on post-thaw sperm motility and viability was reported when semen was frozen in egg yolk-based diluents.[3,8-11] In this study, a liposome containing diluent was used. It is thought that liposomes modify sperm membranes by exchanging lipids and cholesterol, lowering the lipid phase transition temperature and improving cryotolerance.[6] How liposomes interact with seminal plasma components to alter plasma membrane stability is not known. The seminal plasma components that increase sperm sensitivity to cold shock originate from the seminal vesicles.[10] Because electroejaculation results in a higher contribution of seminal vesicle secretions than collection with an artificial vagina,[12] it seemed plausible that removal of seminal plasma would improve post-thaw motility of electroejaculated spermatozoa. However, this hypothesis was rejected in studies in which egg yolk-free diluents were used, including the present one.[4]

As mentioned, there was an interaction between centrifugation and equilibration time on some sperm motion parameters. The lowest values for DSL, VSL, WOB, LIN and STR were observed in centrifuged samples equilibrated for 24h. This is contrary to a previous report in which some of these parameters increased after removal of seminal plasma.[4] However, seminal plasma was removed by filtration and equilibration was limited to 4h. It is possible that centrifugation caused sublethal mechanical damage to spermatozoa. While the damage was not immediately evident, extending the equilibration time could have resulted in further sperm damage and decreased cryotolerance. Because VSL, STR and LIN are positively correlated with bull fertility,[13] the centrifugation protocol used in this study is not recommended if the equilibration time will be extended to 24h.

Previous studies also found an improvement in post-thaw semen quality after equilibration for 24h in non-centrifuged semen.[1,4] No beneficial effect of a prolonged equilibration was observed in this study. Differences in breed, semen collection methods and semen freezing protocols may account for the discrepancy. Moreover, transient exposure of semen to less controlled environmental and handling conditions at the farm could result in sublethal damage that may affect sperm survival during prolonged exposure to refrigeration temperatures. This may counteract the beneficial effect of prolonged equilibration. Despite the lack of a benefit on semen quality, extending the equilibration time did not have a negative effect in non-centrifuged semen and may therefore be beneficial relative to time management in a busy ambulatory practice. This would also allow for semen to be shipped to a semen processing center after on-farm collection for further processing and freezing.

Membrane integrity and sperm apoptosis were not affected by centrifugation or equilibration time. However, acrosome integrity was negatively affected by removal of seminal plasma by centrifugation. Similar findings were reported when seminal plasma was removed by centrifugation in bulls.[4] It is possible that centrifugation caused mechanical damage to the sperm plasma and acrosome membranes.[4] However, acrosomal damage was also reported when seminal plasma was removed by filtration.[4] Seminal plasma contains proteins that stabilize the sperm plasma membrane and prevent premature capacitation. Cryopreservation induces capacitation-like changes in bovine spermatozoa[14] and removal of seminal plasma could render spermatozoa more susceptible to cryocapacitation. This could lead to premature and spontaneous acrosome reaction. Because acrosome response to centrifugation was not evaluated in fresh semen in the first experiment, a second experiment was performed to determine if the acrosomal changes seen were observed immediately after centrifugation and prior to freezing. Acrosome integrity did not differ with centrifugation in fresh semen, possibly indicating that the

differences seen in frozen semen were due to a higher susceptibility to cryocapacitation rather than direct damage caused by centrifugation.

Lastly, freezing semen in the neck of the tank yielded a post-thaw motility, membrane integrity, apoptosis and acrosome integrity similar to traditional vapor freezing. However, acrosomal damage was higher than in non-centrifuged samples frozen traditionally. Holding straws vertically may have delayed the freezing curve of spermatozoa on the top of the straws, which were in a warmer region of the liquid nitrogen vapor, allowing for acrosomal damage or cryocapacitation to occur.

Post-thaw motility in this study was lower than previously reported. Total and progressive post-thaw motility of electroejaculated spermatozoa frozen in BotuBov was 54% and 41%, respectively.[4] Semen diluted in OptiXcell® had a post-thaw motility between 56% and 66%.[6,15] The difference could be due to the use of beef bulls that were not selected for their freezing ability or optimal semen quality in this study. However, since semen was collected at the farm, it was exposed to uncontrolled ambient conditions like temperature, humidity, sunlight and dust. How these conditions affected post-thaw semen quality is unknown. An attempt to control ambient conditions was made by maintaining semen warm within a water bath, working under the shade, in the cleanest possible place, and in the most time-efficient manner. Another difference between this study and previous ones was the use of a manual freezing method instead of a programmable freezer. Although manual and automated methods were not compared here, it can be speculated that the uncontrolled freezing curve may have also decreased post-thaw semen quality.

Based on the findings of this study, a recommended protocol for semen freezing under ambulatory conditions would involve collecting semen using electroejaculation, diluting the semen with the extender immediately after collection, and initiating refrigeration of the diluted semen at the farm. Extending the equilibration time had no effect on the parameters evaluated as long as centrifugation was not performed. Therefore, equilibration of non-centrifuged semen could safely take place while semen is being transported to the laboratory for up to 24h, where the freezing process could be completed using a programmable freezer. Removal of seminal plasma by centrifugation had a negative effect on acrosome integrity. Therefore, centrifugation of electroejaculated bovine semen extended in OptiXcell® at 800 x g for 10 min is not recommended for cryopreservation.

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