Cryopreservation of epididymal and electroejaculated bull semen using liposome- and egg yolk-based extenders

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Abstract

Objective of this study was to compare cryosurvival of epididymal (EP) and electroejaculated (EE) sperm cryopreserved in liposome- or egg yolk- based extenders. Aliquots of EE sperm (n = 10 bulls) were cryopreserved with a liposome- (Optixcell; EEO) or egg yolk-based extender (Botubov; EEB). Bulls were castrated and sperm from each cauda epididymis were cryopreserved with Optixcell (EPO) or Botubov (EPB). Postthaw total motility was higher in semen frozen in Botubov than Optixcell ($p = 0.029$). In addition, sperm distance and velocity parameters, and lateral head displacement were higher in semen frozen in Optixcell ($p = 0.01$). Plasma and acrosomal membrane integrity were higher with Botubov (EEB 60 ± 3%, EPB 59.7 ± 4%; mean ± SEM) than Optixcell (EEO 37.8 ± 6%, EPO 25.8 ± 6%) ($p < 0.0001$). Capacitated cell percentage was lower with Botubov (EEB 2 ± 0.4%, EPB 0.9 ± 0.3%) than Optixcell (EEO 6.9 ± 2.4%, EPO 4.2 ± 1.4%) ($p < 0.0001$). Fewer EP sperm had high mitochondrial potential (EPB 6.6 ± 4.4%, EPO 14.6 ± 6.2%) than EE sperm (EEB 46.7 ± 9.8%, EEO 41 ± 14.6%) ($p = 0.0003$), with no difference between extenders.

Keywords: Bull, freezing, semen, diluents, cryotolerance

Introduction

Egg yolk-based semen extenders have been the industry standard for cryopreservation of bovine semen for many years.[1] However, there are growing concerns about the use of egg yolk, due to potential for microbial contamination, bacterial endotoxin release, presence of steroid hormones and lack of quality standards since egg-yolk composition and viral and bacterial contamination, are often not evaluated.[2] Liposome-based semen extenders are becoming acceptable alternatives to animal-based products.[1] The cryoprotective mechanism of egg yolk is through sequestration of seminal plasma proteins by low-density lipoproteins, reducing cholesterol and phospholipid effluxes and preventing increases in membrane fluidity.[3,4] Since seminal plasma proteins secreted by accessory sex glands are not present in epididymal samples, it would be relevant to determine the response of epididymal sperm to cryopreservation in egg-yolk based extenders. Although the cryoprotective mechanism of liposomes has not been determined, it has been suggested to be mediated via modification of plasma membrane composition by incorporating molecules of interest into the plasma membrane, or exchanging lipids and cholesterol.[5]

Many studies were conducted to determine an ideal semen extender for bull semen cryopreservation, but only a few studies compared egg yolk-based and liposome-based extenders to cryopreserve bovine semen.[6,7] In those studies, liposome-based extenders supported higher or similar postthaw motility of ejaculated sperm than egg yolk-based extenders but lower antioxidant protection.[6,7] However, those studies were carried out with dairy bulls collected with an artificial vagina under controlled laboratory conditions.[6,7] On farm semen collection for cryopreservation from beef bulls typically requires electroejaculation, which yields samples with variable concentrations of seminal plasma. Apparently, no studies have compared egg yolk-based and liposome-based extenders to freeze semen from *Bos taurus* beef bulls collected via electroejaculation versus epididymal harvest.

Assessment of epididymal sperm provides opportunity to study the effects of semen extenders on cryosurvival, as this type of sample contains only seminal plasma proteins from epididymides. Epididymal sperm also have a larger proportion of cytoplasmic droplets when compared to ejaculated samples.[8] These differences may influence sperm response to cryopreservation and their interaction with semen extenders. Bovine epididymal sperm has been routinely cryopreserved using egg yolk-based extenders,[9-17] but studies testing liposome-based extenders for cryopreservation of epididymal sperm are lacking. In addition, most protocols for epididymal sperm freezing were developed for

Bos indicus or Holstein bulls.[9-14] Cryopreservation of epididymal sperm from beef bulls of *Bos taurus* breeds has faced disappointingly low success, with postthaw total motility ranging from 10 to 16%.[15,16] Thus, there is a critical need to improve cryopreservation protocols for these breeds to improve postthaw semen quality, and use of a different type of semen extender may improve postthaw semen quality. Collection and cryopreservation of epididymal sperm is often performed at postmortem to preserve genetics of valuable bulls that face terminal disease or sudden death. Obtaining acceptable postthaw motility is critical to provide acceptable pregnancy rates with epididymal sperm.

Electroejaculation may provide an alternative for collection and preservation of semen from genetically valuable bulls with a terminal disease or sudden death, or for bulls in stud centres refusing to mount a teaser. Ejaculated sperm were reported to have similar postthaw motility and acrosomal integrity, less intact membranes, and higher in vitro fertilization rates than epididymal sperm.[10,11,14] Objective of this study was to evaluate functional sperm parameters associated with cryotolerance in epididymal and electroejaculated sperm frozen with 2 semen extenders. Based on studies conducted in dairy breeds and *Bos indicus* bulls, and on authors' experience, it was hypothesized that there are no differences in response to cryopreservation between electroejaculated and epididymal sperm in beef bulls under the conditions of our study. It was also hypothesized that the liposome-based extender is superior to an egg yolk-based extender for ejaculated and epididymal sperm, due to differences in cryoprotective mechanisms.

Materials and methods

Bulls

Bulls were cared for in accordance with guidelines from the Guide for the Care and Use of Agricultural Animals in Research and Teaching. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Illinois in Urbana-Champaign (Protocol # 17240). Ten Simmangus beef bulls (*Bos taurus*) were available for the study at the University of Illinois. They were housed in pens at the University of Illinois Beef Unit in Urbana, IL. Bulls were kept in groups of 3 per pen and fed a total mixed ratio (63.4% dry matter, 90.7% organic matter, 7.9% crude protein, 55.8% neutral detergent fiber, 32.7% acid detergent fiber, 1.7% ether extract). Bulls were 15.1 ± 0.05 months old (range 14 - 16), weighed 585 ± 17 kg (range 522.7 - 679.6), and had a body condition score of 5.8 ± 0.2 in a scale of 1 - 9 (range 5 - 9) (mean ± SD). Scrotal circumference was measured with a scrotal tape at the widest point of the scrotum. The average scrotal circumference was 40.5 ± 0.6 cm (range 36 - 42).

Experimental design

One ejaculate was collected from all bulls using electroejaculation. After semen evaluation, each ejaculate was divided into 2 aliquots, which were then cryopreserved with 2 semen extenders. One aliquot was cryopreserved using the liposome-based extender Optixcell (IMV Technologies, Maple Grove, MS; treatment EEO), whereas the other was cryopreserved using the egg-yolk based extender Botubov (Botupharma, Scottsdale, AZ; treatment EEB). Botubov is a TRIS-based medium with 20% egg yolk, aminoacids, sugars, buffers, and surfactant Orvus es paste.[12] Optixcell is free of animal-derived components and contains liposomes with phospholipids for cryoprotection. However, the exact composition of both extenders is proprietary and has not been disclosed. Bulls were castrated 3 days later and sperm were collected from epididymis using a swim-up technique. Sperm from 1 epididymal tail from each bull were collected and cryopreserved with Optixcell (treatment EPO), whereas sperm from contralateral epididymis were collected and cryopreserved with Botubov (treatment EPB). Sperm concentration, motility, and morphology were evaluated prior to cryopreservation. Semen was cryopreserved and stored in liquid nitrogen at -196°C for 6 months until evaluation. After thawing, sperm motility, longevity of sperm motility, and hypoosmotic tests were performed at the University of Illinois. Two straws from each treatment were shipped to the University of Georgia in a dry shipper for evaluation of membrane integrity, membrane fluidity, acrosomal integrity, and mitochondrial potential using flow cytometry. Samples were thawed and processed for evaluation in the Andrology Laboratory of the Department of Large Animal Medicine.

Flow cytometry was performed in the Flow Cytometry Laboratory, a facility shared between the Departments of Large Animal Medicine and Population Health. Sperm kinematics and functional parameters were compared among treatments.

Semen collection with electroejaculation

Electroejaculation was performed with a transrectal 60 mm upright bull probe with electroejaculator set on the programmed cycle (Pulsator IV, Lane Manufacturing Inc., Denver, CO). When an inadequate sample was collected, as determined by low volume or absence of sperm motility, a manual cycle of the electroejaculator was used by an experienced clinician. Spermrich fractions were collected in a 15 ml conical tube.

Each ejaculate was divided into 2 aliquots. One aliquot was extended in Botubov at a 1:1 (v:v) ratio and the other aliquot was extended in Optixcell at similar ratio. Extenders were prewarmed to 37°C prior to mixing with semen. Extended semen was stored in 15 ml conical tubes and placed in an insulated Styrofoam box at room temperature until the arrival at the Illinois Veterinary Teaching Hospital within 30 minutes after collection.

Sperm collection from epididymis

Three days after semen collection with electroejaculation, bulls were castrated using a Newberry knife and an emasculator. Once restrained in chute, bull's rectum was emptied. Perineal area was cleaned and scrubbed for a pudendal block. Lidocaine (10 ml/side) was infused at the level of anus, 5 - 10 cm lateral to the edge of the anus. Thereafter, a spinal needle was inserted deeply parallel through the rectum guided by transrectal palpation. Then, 40 ml of lidocaine were infused. Needle was withdrawn, and rectum was massaged for diffusion of lidocaine near pudendal nerve. Procedure was repeated in similar manner in the opposite side. Scrotum was cleaned and aseptically scrubbed with betadine and 70% isopropyl alcohol. Spermatic cords were blocked with 10 ml of lidocaine. Newberry knife was used to cut the bottom of the scrotum; testes were then exposed via incision to spermatic fascia. Emasculator was applied to spermatic cords for 3 minutes. Then, spermatic cords were transfixed and ligated twice before being transected with the emasculator. Spermatic cords were ligated to avoid leakage of sperm. Testes and epididymides were placed in a plastic bag, covered with sterile 0.9% sodium chloride solution at room temperature and placed in a Styrofoam box at room temperature until arrival at the Illinois Veterinary Teaching Hospital within 30 minutes. Upon arrival, cauda epididymides were dissected away from the testis and rinsed with 0.9% sodium chloride solution. Cauda epididymides, including at least 3 cm of the proximal ductus deferens, were separated with a #10 scalpel blade and transferred to a Petri dish. Connective tissue containing superficial blood vessels around the cauda epididymis was removed by dissection with scissors. Tissue was minced with a scalpel blade and covered with 10 ml of warm semen extender (Botubov or Optixcell). After incubation at room temperature for 10 minutes, the medium was aspirated and used for cryopreservation. One epididymis from each bull was processed with Optixcell, and the other with Botubov.

Cryopreservation

After initial dilution, sperm concentration in each aliquot of electroejaculated and epididymal samples was determined using a Nucleocounter (ChemoMetec Inc., Bohemia, NY). Each aliquot was further diluted with the corresponding extender to achieve a final concentration of 40×10^6 sperm/ml. Semen was loaded and sealed into 10 French straws (0.5 ml), with a fully automated machine (MPP Uno, Minitube, Germany) in a cold-room at 5°C. Straws were then equilibrated at 5°C for 3 - 5 hours. After equilibration, straws were placed horizontally on a rack, 5 cm above liquid nitrogen for 15 minutes. Then, straws were plunged in liquid nitrogen, loaded into 10 mm goblets, and transferred to storage in liquid nitrogen tank. Six months after freezing, straws from each aliquot were thawed in a water bath at 38°C for 30 seconds for evaluation of postthaw semen quality.

Semen analysis

At the University of Illinois, semen volume was determined using a graduated 15 ml centrifuge tube. Sperm motility was evaluated with a computer assisted sperm analysis (SpermVision, Minitube of America, Verona, WI) prior to freezing. Semen was placed in a 20 µl sperm analysis chamber over the heated specimen stage at 38°C. Motion parameters were assessed from all cells present in 7 fields with an X 20 phase-contrast objective. Parameters analyzed included total motility (TM, %), progressive motility (PM, %), average path velocity (VAP, µm/second), straight line velocity (VSL, µm/second), curvilinear velocity (VCL, µm/second), average path distance (DAP, µm), curvilinear path distance (DCL, µm), straight line distance (DSL, µm), straightness of the average path (STR), linearity of the curvilinear path (LIN), and amplitude of lateral head displacement (ALH, µm). Default manufacturer's setting for the assessment of bovine semen was used: frame capture speed rate 60 Hz; cell size (min/max) 18/60 μm^2; threshold straightness 50%; VAP cutoff 56 µm/second; and VSL cutoff 28 µm/second. After freezing, 1 straw was thawed from each treatment at 38°C for 30 seconds. Semen was placed in 2 ml tubes in a water bath at 38°C for 4 hours and sperm motility was evaluated every 30 minutes for 4 hours.

Phase contrast microscopy was used for assessment of sperm morphology. A single operator performed all evaluations. Sperm were diluted 1:10 (v:v) with formalin buffered solution and a 5 µl drop was placed on a microscope slide. Evaluation was done at 100 x magnification under oil immersion and 100 sperm were classified based on morphological characteristics. Following the new guidelines from the Society for Theriogenology regarding the classification of sperm morphology, sperm with distal droplets were considered normal.[18]

At the University of Georgia, 1 or 2 straws were thawed from each treatment. Sperm were washed in warm Dulbecco's Phosphate Buffered Solution by centrifugation at 600 x g for 5 minutes to remove semen extender. One aliquot was used for evaluation of mitochondrial potential using the fluorescent probe 5,59,6,69-tetrachloro-1,19,3,39-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1, Cat. No. T3168, Molecular probes, Eugene, OR). One microgram of JC-1 was added to 1 ml of sperm suspension containing 5 x 10^6 sperm, together with 8 µl of a 1.2 mM solution of propidium iodide (PI). Samples were incubated at 38°C for 15 minutes and evaluated under fluorescence microscopy. One hundred membrane-intact sperm (PI negative) were classified as having high (orange) or low (green) mitochondrial potential. Another aliquot was used for evaluation of membrane fluidity using the fluorescent dye merocyanine 540 (M540, Cat. No. M25471, Molecular Probes). The vital stain Yo-Pro 1 (Cat. No. Y3603, Molecular Probes) was added to identify viable cells. Merocyanine 540, 1.5 µl of a 1mM solution in DMSO and 1 µl of a 25 µM solution of Yo-Pro 1 in DMSO were added to a 1 ml suspension containing 5 x 10^6 sperm. Cells were incubated for 10 minutes at 38°C. Using flow cytometry, cells were classified as membrane-intact capacitated sperm (MIC, Yo-Pro 1 negative and M540 positive), membrane-damaged capacitated sperm (MDC, Yo-Pro 1 positive and M540 positive), membrane-intact noncapacitated sperm (MINC, Yo-Pro 1 negative and M540 negative), and membrane-damaged noncapacitated sperm (MDNC, Yo-Pro 1 positive and M540 negative). A final aliquot was used for evaluation of acrosome integrity using peanut agglutinin labelled with fluorescein isothiocyanate (FITC-PNA; Cat. No. F-2301-1, EY Laboratories, San Mateo, CA). For this, 1 µl of a 1 µg/ml solution of FITC-PNA and 1 µl of a 2.4 mM solution of PI were added to a 1 ml suspension containing 5 x 10^6 sperm. Semen was incubated at 38 °C for 10 minutes. Using flow cytometry, cells were classified as membrane-intact acrosome-intact sperm (MIAI, PI negative and FITC-PNA negative), membrane-intact acrosome-damaged sperm (MIAD, PI negative and FITC-PNA positive), membrane-damaged acrosome-intact sperm (MDAI, PI positive and FITC-PNA negative), or membrane-damaged acrosome-damaged sperm (MDAD, PI positive FITC-PNA positive).

Fluorescence activated flow cytometry was performed at the University of Georgia (Accuri™ C6 Plus, BD Biosciences, San Jose, CA). From each sample, 10,000 events in the forward and 90º light scatter population representing whole sperm were analysed using 0.2 µm filtered 18 mega-ohm ultrapure water as sheath fluid. A gate containing sperm was selected based on dot plot distribution of forward (size) versus side scatter (complexity parameter) to eliminate debris and somatic cells from the analysis. Green and red signals were detected using a 5 m Watt blue argon laser (488 nm) and emission filters (535 ± 30 nm for green and 585 ± 30 nm for red). Baseline background fluorescence

signal was initially evaluated in unstained samples. The control area was marked on unstained samples with < 1% of cells registering as positive for both signals. Compensation for FITC emission into the PI detector or vice versa was done by establishing quadrants on sperm labelled only with PI or FITC, followed by electronic subtraction of the FITC emission into the PI detector and PI emission into the FITC detector. Similar compensation was performed for M540 and Yo-Pro 1. After colour compensation, fluorescence emission data were collected with logarithmic amplification for green fluorescence (FITC and Yo-Pro 1 using FL1 detector) and orange-red fluorescence (PI and M540 using FL3 detector). The control quadrant (lower left, LL) was marked on unstained samples to include < 1% of cells as positive in the upper left (UL), upper right (UR) and lower right (LR) quadrants.

Statistical analysis

Statistical analysis was performed using SAS package (SAS Institute, Cary, NC). Distribution of data was tested for normality using a Shapiro Wilk test. Data not normally distributed underwent logarithmic transformation. Results from normally distributed or transformed data were expressed as mean ± SEM. Because data were balanced, arithmetic and LS means coincided. Means of morphology parameters were compared between ejaculated and epididymal sperm using a paired Student's t-test. Motility and flow cytometry data were analyzed using a mixed model, with bull as random effect, and source, time (when applicable) and extender as fixed effects. Where an effect or interaction of source and extender was identified, pairwise comparisons were performed using least square means. Comparisons among treatments within time points were performed using proc GLM and Tukey's test when an interaction of source, extender and time was identified in the mixed model. Significance was considered when $p < 0.05$.

Results

When new guidelines from the Society for Theriogenology were followed, the percentage of normal sperm did not differ between ejaculated (69.1 ± 4.3%) and epididymal samples (67.8 ± 5.5%). However, when distal droplets and distal midpiece reflexes were counted separately, the percentage of morphologically normal sperm was higher in ejaculated (57.5 ± 5%) than epididymal samples (26.4 ± 4.5%; $p = 0.0005$), due to a lower percentage of ejaculated sperm with distal droplets (EE 11.6 ± 3.6% versus EP 41.4 ± 6.7; $p = 0.002$; Table).

Table: Morphological characteristics of ejaculated and epididymal bovine sperm.

Morphology parameter (%)	Ejaculated sperm (n = 10)	Epididymal sperm (n = 10)
Normal	57.5 ± 5[a]	26.4 ± 4.5[b]
Proximal droplets	10.9 ± 3	12.9 ± 4.7
Distal droplets	11.6 ± 3.6[a]	41.4 ± 6.7[b]
Abnormal heads	2.5 ± 1.2	2.7 ± 0.8
Abnormal midpieces	1 ±0.4	1.6 ± 0.7
Abnormal acrosomes	0.2 ± 0.1	0.1 ± 0.1
Coiled tails	3.2 ± 0.8	1.5 ± 0.8
Kinked tails	6.9 ± 2.2	7.3 ± 2.6
Detached heads	6.2 ± 2.9	5.7 ± 3.1

[a,b]Within a row, means without a common superscript differed ($p < 0.05$)

Total motility was affected by time ($p < 0.0001$), extender ($p < 0.0001$) and their interaction ($p = 0.029$), but not source of sperm. All postthaw samples had lower motility than pre-freeze samples. However, semen frozen in Botubov had higher total motility than in Optixcell immediately postthaw and after 3 hours of incubation ($p < 0.05$; Figure 1A). Progressive motility was affected by time ($p < 0.0001$), but neither by source nor extender (Figure 1A). All postthaw samples had lower progressive motility than prefreeze samples. There was an interaction among extender, time and source in all distance and velocity parameters ($p = 0.01$). In general, ejaculated sperm frozen in Optixcell had higher DAP, DSL, DCL, VAP, VSL, and VCL than other treatments (Figure 1 C - H; $p < 0.05$). Sperm frozen in Botubov had higher STR and LIN, but lower ALH than sperm frozen in Optixcell (Figure1 I - K; $p < 0.05$).

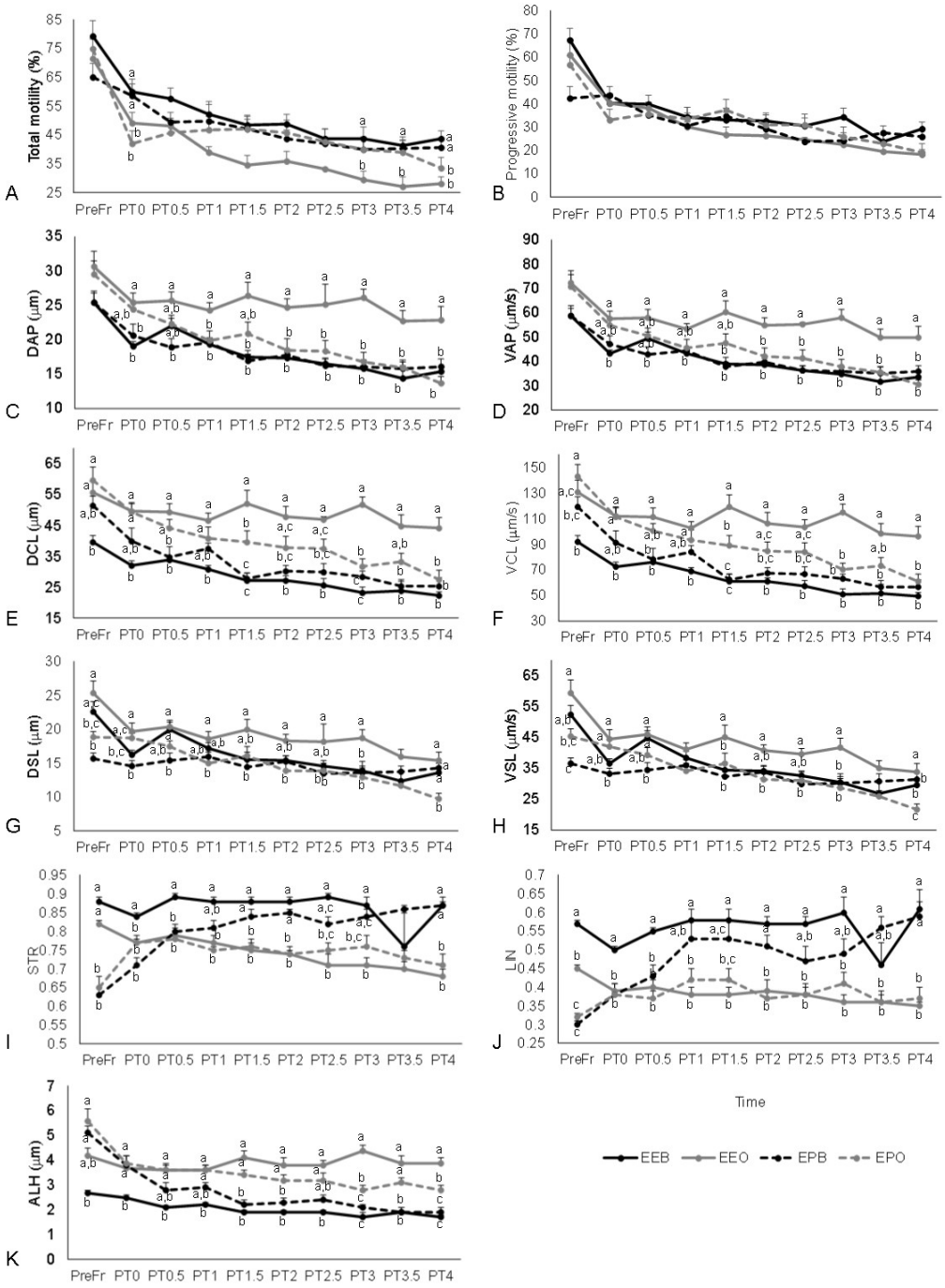

Figure. 1 A - K. Sperm motion parameters before freezing (PreFr), and 0 - 4 hours postthaw (PT0 - PT4) in electroejaculated and epididymal bovine sperm frozen in an egg-yolk (Botubov) or liposome-based (Optixcell) semen extender. EEB = electroejaculated, Botubov; EEO = electroejaculated, Optixcell; EPB = epididymal, Botubov; EPO = epididymal, Optixcell; DCL = curvilinear distance, DAP = distance of average path, DSL = straight line distance, VCL = curvilinear velocity, VAP = average path velocity, VSL= straight line velocity, LIN = linearity, STR = straightness, ALH = amplitude of lateral head displacement. [a-c] Within a time point, means without a common superscript differed ($p < 0.05$).

Percentage of membrane-intact and membrane-damaged cells with capacitation-like associated changes in membrane fluidity differed with extender (IMC $p < 0.0001$; DMC $p = 0.001$), but not source (Figure 2 A - B). There was a lower percentage of capacitated sperm in semen frozen in Botubov than Optixcell. Percentage of sperm with intact membranes and intact acrosomes (IMIA)

was higher in sperm frozen in Botubov than Optixcell ($p < 0.0001$), with no effect of source (Figure 2 C). There was an interaction of extender with source for percentage of sperm with damaged membranes and damaged acrosomes, with electroejaculated semen frozen in Botubov having the lowest value. The other 2 acrosomal parameters (DMIA, IMDA) did not differ with extender or source. Percentage of cells with low mitochondrial potential was higher in epididymal than ejaculated sperm, with no difference between extenders ($p = 0.0003$; Figure 2D), whereas the opposite was true for percentage of sperm with high mitochondrial potential ($p = 0.03$).

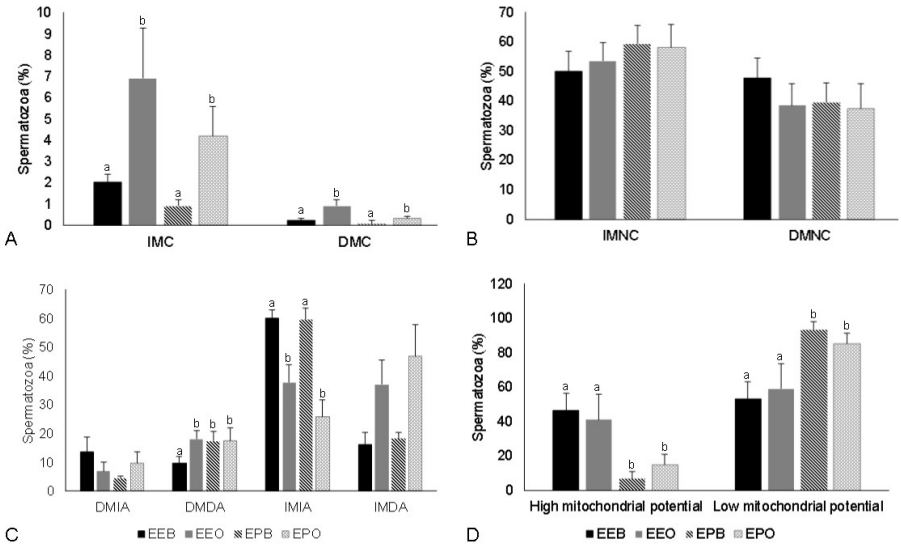

Figure 2 A - D. Percentage of sperm with intact membranes and capacitated (IMC), damaged membranes, capacitated (DMC) (panel A); intact membranes, noncapacitated (IMNC), damaged membranes, noncapacitated (DMNC) (panel B); damaged membrane, intact acrosome (DMIA), damaged membrane, damaged acrosome (DMDA), intact membrane, intact acrosome (IMIA), intact membrane, damaged acrosome (IMDA) (panel C); and with high and low mitochondrial potential (panel D) in electroejaculated and epididymal bovine sperm frozen in an egg-yolk (Botubov) or liposome-based (Optixcell) semen extender. EEB = electroejaculated, Botubov; EEO = electroejaculated, Optixcell; EPB = epididymal, Botubov; EPO=epididymal, Optixcell. [a,b]Within an end point, means without a common superscript differed ($p < 0.05$).

Discussion

The ability of 2 extenders to support cryosurvival of epididymal and ejaculated beef bull sperm was assessed in this study. Based on in vitro sperm parameters, we inferred that egg-yolk based extender (Botubov) provided superior cryoprotection compared to the liposome-based extender (Optixcell) in electroejaculated semen from beef bulls. Postthaw motility was higher in semen frozen in an egg-yolk based extender than sperm frozen in the liposome-based extender, regardless of sperm source. Although markers for oxidative stress were not assessed herein during incubation at 38ºC, we inferred that egg-yolk based extender was superior to control oxidative stress during the thermal longevity test. Findings of a previous study that compared liposome- and egg yolk-based extenders to cryopreserve bull semen appeared to support this suggestion, as sperm frozen in egg-yolk based extender had lower production of intracellular superoxide compared to sperm frozen in liposome-based extender.[7]

This was apparently the first study comparing the liposome-based extender Optixcell and the egg yolk-based extender Botubov to cryopreserve electroejaculated semen or epididymal sperm from beef bulls. Previous studies with dairy bulls ejaculated into an artificial vagina concluded that whereas a liposome-based extender yielded higher or similar postthaw motility than an egg yolk-based extender, antioxidant protection was lower with the liposome-based extender.[6] Other authors reported higher postthaw motility and fertility of buffalo semen frozen in Optixcell than Tris-egg yolk extender.[19-21] Although acrosomal and membrane integrity were higher with Optixcell in 1 study,[19] there was no difference between extenders in 2 other studies.[20,21] Apparent differences in findings may be explained by breed or species differences in seminal plasma composition, as well as

differences in seminal plasma contribution or composition associated with semen collection methods. Furthermore, the egg yolk-based extender Botubov is supplemented with amino acids and sugars beneficial for cryopreservation of bovine semen. Although the formulation is proprietary, presumably composition of the extender used also accounted for the differences in findings between this study and previous ones. Furthermore, Botubov was superior to traditional TRIS-egg yolk extenders for freezing sex-sorted semen,[22] perhaps due to Botbov's apparently more complex composition (e.g. amino acids).

Sperm frozen in liposome-based extender had higher values of distance and velocity parameters and lateral head displacement and lower straightness and linearity than sperm frozen in the egg yolk-based extender. This motility pattern has been associated with sperm capacitation.[23] Capacitated sperm have a change in their motion pattern called hyperactivation; distinguishing characteristics are increased VCL and ALH, and decreased LIN.[23] Indeed, sperm frozen in liposome-based extender had a higher percentage of cells with capacitation-associated membrane fluidity changes. Cryopreservation induces a significant loss and alteration of phospholipids and cholesterol in the sperm membrane.[23] Loss of cholesterol is a key event in capacitation, leading to premature capacitation in cryopreserved sperm.[24,25] Moreover, changes in cholesterol/phospholipid ratio, or loss of decapacitating proteins, increase sensitivity of cryopreserved sperm to capacitating agents.[24,25] Additional studies should assess if in vitro findings affect in vivo or in vitro fertility.

Presence of capacitated sperm in frozen thawed bovine semen is correlated to poor field fertility, based on 56-day nonreturn rates after artificial insemination.[26,27] Therefore, ability of semen extenders to prevent cryocapacitation is important in providing appropriate cryosurvival. As ejaculated sperm are mixed with secretions from accessory sex glands, the sperm plasma membrane is subjected to interactions with various molecules, particularly proteins, present in secretions from these glands.[28] Interestingly, percentage of sperm with intact membranes and intact acrosomes was higher in both epididymal and ejaculated sperm frozen in Botubov than Optixcell, suggesting that the egg-yolk based extender was a superior option regardless of the presence of seminal plasma. Sperm frozen in the liposome-based extender also had numerically higher percentage of damaged acrosomes, although the difference was only significant in ejaculated semen, likely due to the low number of animals used. The lower percentage of sperm with intact acrosomes could represent poorer preservation of acrosomal integrity during freezing and thawing, or a higher prevalence of spontaneous acrosome reactions associated with cryocapacitation when a liposome-based extender was used.

Seminal plasma contains a family of closely related proteins, collectively called binder of seminal plasma proteins (BSP). These proteins bind to the sperm membrane choline phospholipids at ejaculation and potentiate sperm capacitation by stimulating cholesterol and phospholipid efflux from the sperm membrane.[28] Egg yolk lipoproteins specifically bind to and scavenge BSP proteins, preventing their interaction with the sperm plasma membrane.[28] Thus, egg yolk lipoproteins reduce cholesterol and phospholipid efflux and reduce sperm susceptibility to cryocapacitation.[3] In addition, sperm membrane also incorporates cholesterol and phospholipids from egg yolk lipoproteins, increasing their content during incubation.[2] Although epididymal sperm lack exposure to seminal plasma, egg yolk might have increased sperm cryotolerance through direct modification of the sperm plasma membrane composition. However, this warrants further studies. Although liposomes also sequester BSP proteins with a similar efficiency to egg yolk, incorporation of lipids into the plasma membrane of sperm cells is lower and less efficient when liposomes are used.[29] This may in part explain the better cryoprotective results obtained with the egg yolk-based extender when compared to liposome-based extender, even in epididymal sperm (that lacks seminal plasma exposure).

Reports comparing freezing ability of bovine epididymal and ejaculated sperm are scarce and sometimes contradictory. Although sperm motility did not differ, epididymal sperm had more intact membranes after freezing/thawing than ejaculated sperm.[10,11] Whereas acrosome integrity was similar in 1 study, more acrosomal damage was reported in epididymal sperm in another study.[10,11] Although 1 study reported similar percentage of cleaved embryos on day 3 and blastocyst development on day 7 after in vitro fertilization[8], lower cleavage rate (cleaved embryos on day 3/mature oocytes) was reported with frozen epididymal sperm than ejaculated sperm in another study.[14] However, in the current study, there were no differences between ejaculated and epididymal sperm in postthaw motility, plasma and acrosomal membrane integrity, or membrane fluidity. High percentage of

proximal and distal droplets observed in epididymal sperm in this study were expected, as bulls enrolled in this study were young and epididymal sperm have a high percentage of cytoplasmic droplets, although many are shed during ejaculation.

Regardless of the semen extender used, frozen/thawed epididymal sperm had a remarkably lower mitochondrial potential than ejaculated sperm in this study. Cytoplasmic droplets are enriched for enzymes involved in energy production and phosphorylation.[30] Perhaps the higher percentage of cytoplasmic droplets in epididymal sperm resulted in higher energy and ROS production, thus leading to mitochondrial damage. Conversely, mitochondrial potential increases during transit through the epididymis.[29] Mitochondrial potential was not evaluated herein before freezing, but it is possible that differences exist between epididymal and ejaculated sperm mitochondrial function. Moreover, the association between mitochondrial function in epididymal and ejaculated sperm and fertility is not known.

Conclusion

Contrary to expectations, the egg yolk-based semen extender studied herein provided better cryoprotection than liposome-based extender, with a higher protective action on sperm motility, membrane and acrosome integrity, and membrane fluidity. Epididymal sperm had higher or similar postthaw motility, acrosome integrity or membrane fluidity than ejaculated sperm. However, mitochondrial potential was lower. It remains to be determined if superior in vitro results obtained with an egg yolk-based extender translates into better in vivo and in vitro fertility when compared to a liposome-based extender.

Conflict of Interest

Authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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