Semen extender type and cold storage of tissue affect cryosurvival of alpaca epididymal sperm

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Abstract

The objectives of this study were to determine the ability of liposome- and DMFA-containing semen extenders to support cryosurvival of alpaca epididymal sperm, and to evaluate the effect of epididymal cold storage on post-thaw viability. It was hypothesized that both semen extenders provide appropriate cryoprotection, and post-thaw sperm viability does not differ when alpaca epididymal sperm are cryopreserved immediately after castration or after 24 h of cold storage. Sperm were recovered from one epididymis from each male (n = 10) on the day of castration, and from the other one after 24 h of tissue storage at 4°C. On each day, each sample was divided into two aliquots, which were cryopreserved in semen extender OptiXcell® or BotuCRIO. Pre-freezing sperm parameters did not differ between samples obtained on the day of castration or 24 h later. Motility of sperm cryopreserved in BotuCRIO on the day of castration did not differ from pre-freezing values. Use of OptiXcell® or delaying sperm cryopreservation decreased total ($p = 0.002$) and progressive sperm motility ($p = 0.041$) compared with pre-freezing values seen in sperm recovered on the day of castration. Membrane integrity was lowest when sperm were cryopreserved in OptiXcell® after cold storage. Treatment had no effect on DNA integrity. It was concluded that the DMFA-containing semen extender BotuCRIO was superior to the liposome-containing semen extender OptiXcell® at preserving post-thaw viability of alpaca epididymal sperm. Cold storage of epididymides for 24 h negatively affected post-thaw motility and membrane integrity of sperm. Therefore, cryopreservation with BotuCRIO immediately after castration is recommended for best preservation of post-thaw viability of alpaca epididymal sperm.

Keywords: Camelid, male, diluent, freezing, post-thaw

Introduction

Harvest and cryopreservation of epididymal sperm enables preservation of genetic material in the event of unexpected death of an animal. While reports of this technique exist in Camelids, an efficient protocol for epididymal sperm cryopreservation has not been developed. Initial reports have shown that lactose-based semen extenders provided better post-thaw motility than citrate- or TRIS-based extenders.[1] Using lactose-based extenders, it was then shown that final concentrations of 3 to 4% glycerol and 1% Equex STM paste provided ideal cryoprotection.[2] However, mean post-thaw motility was low, ranging from 14.4 to 27%.[1,2] Later work testing alternative cryprotectants demonstrated that post-thaw motility was improved when dimethylsulfoxide (DMSO) or diacetylamide (DMA) were used instead of ethylene glycol or glycerol.[3,4] With addition of these cryoprotectants, mean post-thaw motility ranged from 31 to 34%.[3,4] Amides, like DMA and dimethylformamide (DMFA) are small hydrophilic molecules with better cell penetration and less toxicity than glycerol.[4,5] Adding DMFA to the semen extender improved cryosurvival of stallion epididymal sperm.[6] In bulls, addition of liposomes improved post-thaw semen quality.[7,8] Liposomes can incorporate into the plasma membrane and change the lipid phase transition, decreasing the sensitivity of sperm to low temperatures.[7] The use of liposome and DMFA containing commercial semen extenders for cryopreservation of alpaca sperm has not been yet tested.

In addition to semen extenders, holding time could also have an impact on post-thaw semen quality. The ability to hold epididymides for a prolonged time allows practitioners to transport tissue to the laboratory for further processing and cryopreservation. This practice is routinely performed in stallions since cold storage for up to 96 h does not affect quality or fertility of cryopreserved equine sperm.[9,10] In spite of this apparent advantage, there have been no direct comparisons on post-thaw motility of alpaca epididymal sperm cryopreserved immediately after castration or after 24 h of cold storage.

The objectives of this study were to determine the ability of commercial semen extenders containing liposomes and DMFA to support cryosurvival of alpaca epididymal sperm, and to evaluate the effect of cold storage on post-thaw sperm parameters. It was hypothesized that both semen extenders provide appropriate cryoprotection, and that post-thaw sperm parameters do not differ when alpaca epididymal sperm are cryopreserved immediately after castration or after 24 h of cold storage.

Materials and methods

Animals and castrations

Twenty testes were collected from 10 male alpacas. The alpacas were of Huacaya and Suri breeds, and 1 to 7 y old. They were presented to the Veterinary Medical Center of the University of Georgia for elective castration. The reproductive history of these males was not known. The alpacas were anesthetized with xylazine (0.4 mg/kg), ketamine (4 mg/kg) and butorphanol (0.04 mg/kg), all of them given intramuscularly. The testes and epididymides were removed using a standard closed castration technique. Prior to castration, the deferent ducts were ligated with 2 PDS II. Each pair of testes with epididymides was placed in a clean whirlpack bag without any addition of media, and was placed at 4°C in the truck's refrigerator until processing.

Experimental design and sperm collection

Consequent to arrival to the laboratory, one epididymis from each male was immediately processed, while the other testis remained at 4°C until processing the following day. Tissue was processed as previously described, with some modifications.[1,2] The parietal vaginal tunic was opened, and the testes and epididymides were exposed and rinsed with 0.9% sodium chloride solution. A ligature (2-0 PDS II) was placed at the junction between the corpus and cauda epididymides. Cauda epididymides, including at least 1 cm of the proximal ductus deferens, were separated and transferred to a Petri dish. Connective tissue containing superficial blood vessels around the cauda epididymis was removed by dissection with scissors. The tissue was then covered with fractionated milk-based semen extender (INRA96, IMV Technologies, Maple Grove, MN, USA), and minced into small fragments with a scalpel. While there is no information in alpacas, the use of INRA96 resulted in better preservation of membrane integrity and fertility of liquid-stored camel semen compared with other commercial extenders.[11] Sperm were allowed to swim up into the medium by incubation for 15 min at 38°C. After incubation, the tissue fragments were removed with forceps. The medium containing sperm was filtered through a stallion semen gel filter (Max-Flow semen gel filters, Nasco, Ft. Atkinson, WI, US) into a 15-ml tube to remove small tissue fragments and debris. A complete semen evaluation was performed as described below, including evaluation of volume, sperm concentration, morphology and motility. Then, each sample was divided into two aliquots. Both aliquots were centrifuged at 900 x g for 10 min. This centrifugation force has no negative effects on sperm motility or viability, and minimizes sperm losses in the supernatant.[12] After discarding the supernatant, one aliquot was resuspended with the liposome-containing semen extender OptiXcell® (IMV Technologies, Maple Grove, MN, USA) to dilute sperm to a final concentration of 25 x 10^6/ml. There are no reports in the literature on the ideal sperm concentration for alpaca semen cryopreservation. The concentration used was selected to enable the division of the samples into the number of aliquots needed. Sperm diluted in OptiXcell® were placed in the refrigerator at 4°C for 5 h for equilibration. After equilibration, pre-cooled 0.5 ml French straws were loaded. The other centrifuged aliquot was diluted with the DMFA-containing semen extender BotuCRIO (Botupharma USA, Phoenix, AZ, USA) until a final sperm concentration of 25 x 10^6/ml was reached. Sperm diluted in BotuCRIO were loaded into 0.5-ml French straws at room temperature, and equilibrated at 4°C for 15 min. Next, all straws were placed horizontally on a rack, 3 cm above liquid nitrogen for 15 min. The straws were plunged in liquid nitrogen, loaded into 10-mm goblets, and transferred to the storage tank. All straws were stored at -196°C until evaluation. The cryopreservation protocols used were based on instructions from the manufacturer of each semen extender. The other epididymis from each male was maintained in the plastic

bag at 4°C for 24 h. After 24 h of cold storage, the cauda epididymides were processed as described for the contralateral testis. Each sample was also divided into two aliquots, diluted with OptiXcell® or BotuCRIO, and cryopreserved following the same protocols as described above. Two weeks after freezing, one straw from each aliquot was thawed in a water bath at 38°C for 30 s, and sperm motility and membrane integrity were evaluated. Another straw was thawed for evaluation of DNA integrity.

Semen evaluation

Immediately after recovery of sperm, sample volume was determined using a graduated 15-ml centrifuge tube. Sperm concentration and motility were evaluated with a computer assisted semen analyzer (CASA; SpermVision Professional, Minitube of America, Verona, WI, USA). The settings of the instrument were: Field depth of view 20 μm, pixel to μm ratio 130 to 100, cell area 18 to 80 μm, frames acquired 30, frame rate 60 Hz, AOC cut off static cells 5 and DSL cut off 4.5 μm/s (progressive motility). Semen was placed in a 20-μL sperm analysis chamber over the heated specimen stage at 38°C. Mean percentage of total (TMOT) and progressively (PMOT) motile sperm was assessed from all cells present in seven fields with a X 20 phase-contrast objective. Phase contrast microscopy was used for assessment of sperm morphology. 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 100x magnification under oil immersion, and 100 sperm were classified based on their morphological characteristics. Two weeks after freezing, one straw from each aliquot was thawed in a water bath at 38°C for 30 s, and sperm motility and membrane integrity were evaluated using CASA. In addition to TMOT and PMOT, other motion parameters analyzed post-thaw 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). Membrane integrity was evaluated using fluorescence microscopy under a 40X objective. Sperm were incubated for 10 min at 38°C with the vital stains SYBR14 and propidium iodide (Live/dead kit, Minitube of America) following instructions from the manufacturer. All sperm presented in 10 fields were classified as membrane-intact (green fluorescence) or membrane-damaged (red fluorescence) by a built-in software within the CASA system (SpermVision Professional™, Minitube of America), and mean percentages were reported.

Sperm chromatin structure was evaluated on frozen-thawed semen using acridine orange and flow cytometry (Accuri™ C6 Plus, BD Biosciences, San Jose CA, USA). Frozen-thawed sperm were washed in phosphate buffer solution by centrifugation at 600 x g for 5 min. After removing the supernatant, the pellet was re-suspended to 0.5 ml in TNE buffer (0.186 g disodium ETDA, 0.79 g Tris-HCL, 4.380 g NaCl in 500 ml deionized water, pH 7.4, 4°C). Then, 15 μl of the resuspended sample was diluted with 185 μl of TNE buffer. This dilution provided a final sperm concentration of 2 x 10^6/ml. Semen was incubated for 30 s after addition of 400 μl of acid-detergent solution (2.19 g NaCl, 1 ml 2N HCl, 0.25 ml Triton X, q.s. 250 ml deionized water, pH 1.2, 4°C). Finally, 1.2 ml of a 2 μg/ml working solution of acridine orange (Invitrogen, Waltham, MA, USA, Cat. No. A3568) in buffer (3.8869 g citric acid monohydrate, 8.9429 g Na_2HPO_4, 4.3850 g NaCl, 0.1700 g disodium EDTA, q.s. 500 ml water, pH 6) was added and semen was incubated for 3 min at room temperature. The sample flow was initiated for 1 min to allow for hydrodynamic equilibration, after which data collection was initiated. The flow rate was adjusted to around 200 cells/sec. From each sample, 20 000 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 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. The green and red signals were detected using a 5mWatt 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. The main population represented sperm that emitted more green than red fluorescence (native double-stranded DNA). Cells located to the right and below the main

population represented sperm with increased red fluorescence and decreased green fluorescence (denatured single-stranded DNA). A modified DNA fragmentation index (mDFI) was calculated as the percentage of sperm with denatured DNA (red) divided by the percentage of total sperm with fluorescence emission (green + red).

Statistical analysis

Statistical analysis was performed using SAS package (SAS Institute, Cary, NC, USA). Distribution of the data were tested for normality using a Shapiro Wilk test. Variables that did not follow a normal distribution underwent logarithmic transformation. Means were compared between treatments using ANOVA, including the fixed effect of treatment. Paired comparisons were made using a Tukey's test. Data were expressed as mean ± SE and differences were considered significant if $p < 0.05$.

Results

Sperm were obtained from 70% (7/10) of the epididymides processed on the day of castration, and 60% (6/10) of those processed after 24 h of cold storage. Pre-freezing sperm parameters did not differ between the samples obtained on the day of castration or 24 h later ($p > 0.05$; Table 1). The proportion of pre-freezing total and progressive motility retained (% of initial motility) after 24 h of cold storage was 65.1±10.9% and 49.9±16.1%, respectively.

Motility of sperm cryopreserved in BotuCRIO on the day of castration did not differ from pre-freezing values. However, total ($p = 0.002$) and progressive ($p = 0.041$) sperm motility were lower when OptiXcell® was used or cryopreservation was delayed for 24 h (Figure). Post-thaw membrane integrity was also affected by treatment and was lowest when sperm were cryopreserved in OptiXcell® after cold storage ($p = 0.039$; Figure). No other sperm motion parameters differed with treatment (Table 2). Similarly, post-thaw mDFI did not differ with treatment. Mean mDFI was 2.5 ± 1.8% and 4.3 ± 3.1% for sperm cryopreserved immediately in BotuCRIO or OptiXcell®, respectively; and 3.3 ± 1.8% and 4.1 ± 3.1% for sperm cryopreserved after cold storage in BotuCRIO or OptiXcell®, respectively.

Discussion

To the authors' knowledge, this is the first study directly comparing post-thaw quality of alpaca epididymal sperm recovered on the day of castration and after 24 h of cold storage. Without direct comparisons between holding times, recommendations for immediate or delayed processing of alpaca epididymides could not be made. While mean sperm motility in this study was not statistically different between storage times, storage of testes with epididymides at 4°C for 24 h resulted in loss of 35% and 51% of total and progressively motile sperm, respectively. Moreover, post-thaw motility was significantly decreased by cold storage compared with pre-freezing values of sperm recovered on the day of castration. While the ability to preserve sperm viability for a prolonged time would allow practitioners to ship the epididymides of deceased males to a centralized facility for sperm recovery and cryopreservation, based on the results of this study, this practice is not recommended in alpacas. This is in contrast with findings in other species. In stallions and bucks, sperm viability and fertility were maintained when epididymides were cooled for 48 to 96 h.[9,10,13] Differences in diluent, cryoprotectant or sperm membrane composition may account for the discrepancies among species. The osmolality of the semen extender was shown to affect survival of cold-stored ram epididymal sperm.[14] It was proposed that since the osmolality of the epididymal medium increases with post-mortem time, epididymal sperm might adapt to an increasingly hypertonic environment, requiring the use of semen extenders with higher osmolality for best response to cryopreservation.[14] The effect of storage time on osmolality of epididymal contents recovered post-castration, and the effect of osmolality of semen extender on post-thaw viability of alpaca sperm require investigation.

This study was also first to evaluate the ability of commercial semen extenders containing DMFA or liposomes to support cryosurvival of alpaca sperm. The use of a diluent containing DMFA provided the best preservation of post-thaw motility. The liposome-containing semen extender was less suitable for

cryopreservation of alpaca epididymal sperm, resulting in decreased post-thaw motility and membrane integrity at both processing times. Adding DMFA to freezing extenders also had a beneficial effect on post-thaw motility of stallion epididymal sperm.[7] This is thought to be because DMFA is a small hydrophilic molecule with good cell penetration and low cytotoxicity.[4,5]

In this study, sperm were recovered from 60 to 70% of the epididymides. This number was lower than previously reported (90-100%). Previous studies have included animals of 2 to 4 y of age and testicular size was a criterion for inclusion in the study. Males in this study were between 1 and 7 y old. No attempt was made to identify the donor of each tissue or measure testicular size. It is possible that the epididymides that yielded no sperm belonged to young peri-pubertal animals. On the other hand, the number of sperm recovered from each male that did yield a sample was higher than previously reported. Reported mean recovery ranged from 75.3 to 143.1 $x10^6$ sperm/male.[1,2] In this study, a mean of 560 $x10^6$ sperm/male (range 203 to 898.5 $x10^6$ sperm/male) was obtained. Pre-freezing sperm motility was comparable to previous reports.[1,2,15] The mean percentage of morphologically normal sperm was 29%. The main abnormality present was cytoplasmic droplets (45%). Cytoplasmic droplets can be present in more than 60% of alpaca sperm in the terminal segment of the cauda epididymis, and are lost when sperm reach the ductus deferens.[16] While these findings represent physiological changes that occur during epididymal transit, the effect of epididymal sperm morphology on alpaca fertility is not known.

In conclusion, a DMFA-containing commercial semen extender (BotuCRIO) was superior to a liposome-containing semen extender (OptiXcell®) at preserving post-thaw motility and membrane integrity of cryopreserved alpaca epididymal sperm. Cold storage of epididymides for 24 h negatively affected post-thaw sperm motility and membrane integrity. Therefore, cryopreservation with BotuCRIO immediately after castration is recommended for best preservation of post-thaw viability of alpaca epididymal sperm.

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Table 1. Pre-freezing parameters of epididymal sperm collected on the day of castration or after 24 h of cold storage. Data are expressed as mean ± SEM.

Parameter	Day of castration	24 h after castration
Total sperm motility (%)	50.1±6.1	34.2±16.1
Progressive sperm motility (%)	33.7±5.7	19.5±6.2
Normal sperm (%)	29.1±5	29.8±6
Abnormal acrosomes (%)	0.8±0.5	0.3±0.3
Abnormal heads (%)	6.1±3.1	7±2.2
Abnormal midpieces (%)	6.7±2.1	3.5±1.1
Kinked tails (%)	7.8±2.3	8.1±3
Proximal droplets (%)	32.5±6.8	38.3±4
Distal droplets (%)	10.8±2.2	9.7±2.3
Coiled tails (%)	4.4±2	2.3±1.2
Detached heads (%)	1.4±1.1	0.8±0.8
Sperm concentration ($x10^6$/ml)	53.6±10.9	71.4±12.5
Sample volume (ml)	6.3±0.3	3.7±0.1
Total sperm ($x10^6$)	326.9±55.9	271.9±54.2
Total progressively motile sperm ($x10^6$)	121.4±28.8	64.9±23.9
Total normal motile sperm ($x10^6$)	28.8±5.8	14.1±4.8

Table 2. Motion parameters of epididymal alpaca sperm after cryopreservation in BotuCRIO or OptiXcell® on the day of castration (0) or after 24 h of cold storage (24). 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, WOB=wobble, BCF= beat cross frequency, ALH= amplitude of lateral head displacement. Data are expressed as mean ± SEM.

Variable	Botucrio0	Optixcell0	Botucrio24	Optixcell24
DCL (μm)	34.5±2	35.8±4.5	34.5±2.4	32.8±5.2
DAP (μm)	17.9±0.9	16.7±0.9	16.9±0.5	15.8±2.1
DSL (μm)	10.5±0.4	10.2±0.4	10±0.4	10.4±0.9
VCL (μm/sec)	75.5±4.5	79.2±8.6	75.6±4.6	70.5±11
VAP (μm/sec)	39.7±2	38.2±1.7	37.4±1.2	34.6±4.7
VSL (μm/sec)	23.5±1.2	23.9±1.2	22.5±1.1	22.8±2.2
LIN	0.31±0.01	0.3±0.04	0.3±0.03	0.34±0.03
STR	0.59±0.02	0.62±0.02	0.6±0.03	0.67±0.02
WOB	0.52±0.01	0.5±0.04	0.5±0.03	0.51±0.04
BCF (hertz)	14.7±0.6	14.9±1.6	14.7±0.5	12.8±1.8
ALH (μm)	3.8±0.3	3.9±0.1	3.5±0.09	3.1±0.4

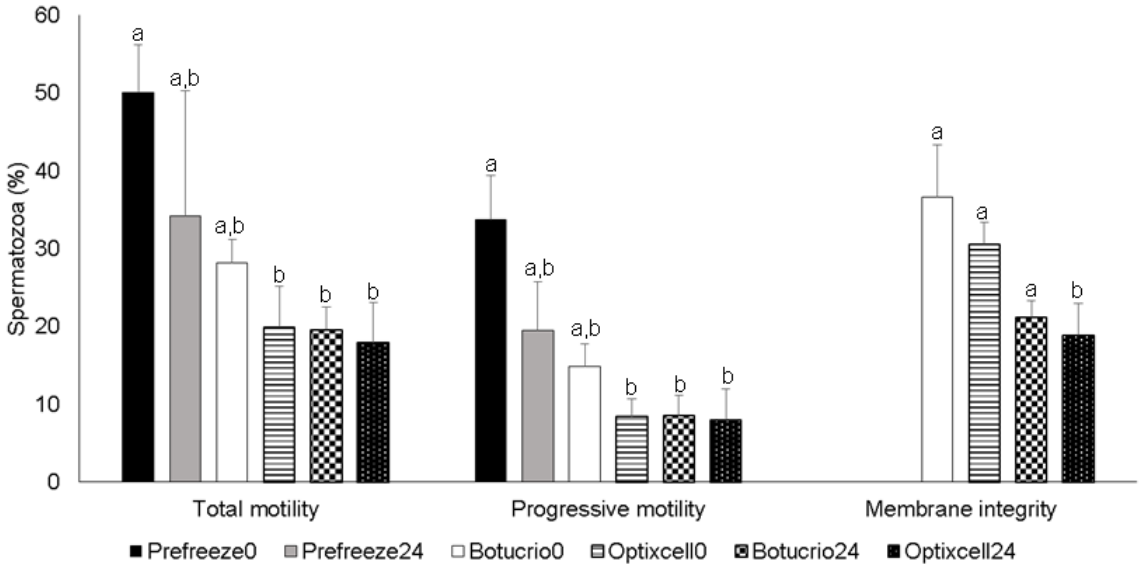

Figure. Motility of alpaca epididymal sperm prior to freezing (Prefreeze), and after cryopreservation in BotuCRIO or OptiXcell® on the day of castration (0) or after 24 h of cold storage (24). Membrane integrity was only evaluated after cryopreservation. Data are expressed as mean ± SEM. [a,b]Indicate differences among treatments ($p < 0.05$).