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Effect of Semen Extender and Storage Temperature on Motility of Ram Spermatozoa

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Abstract

Cryopreservation of ram semen results in low post-thaw survival. Thus, studies were conducted to optimize liquid-storage of chilled semen. In Experiment 1, semen collected from rams (n = 5) was extended using either milk, TRIS (tris[Hydroxy-methyl]aminomethane), TEST (N-Tris (hydroxymethyl)-methyl-2-aminoethane sulfonic acid), or CJ-2 (choline-based extender) and stored at either 4˚C and 15˚C. All extenders were supplemented with 5% (v/v) egg yolk. In Experiment 2, semen collected from 9 rams was distributed across TRIS or milk extenders supplemented with 5% or 20% (v/v) egg yolk, and 0 or 1% ethylene glycol (EG), during storage for 72 hours at 4˚C. The effect of penicillamine, hypotaurine, and epinephrine (PHE) on motility parameters was also evaluated following storage. In Experiment 1, most of the sperm motility parameters were higher after extension, and storage at 4˚C compared with 15˚C ($P < 0.05$). Ram semen extended using milk or TRIS based extenders and stored at 4˚C maintained similar sperm progressive motility over time, but was higher compared with TEST or CJ-2 extenders ($P < 0.05$). However, progressive motility of ram sperm declined by 75% when stored at 4˚C for 24 hours, and continued to decline over time. In Experiment 2, milk extender supplemented with 1% EG and 20% egg yolk before storage and addition of PHE after storage had higher sperm motility parameters than other extender and supplement combinations. Further studies are needed to assess *in vivo* sperm viability and conception rates when using extended, stored semen for artificial insemination of ewes.

Keywords

Semen Extenders, Ram Semen, Liquid Storage, Sheep

1. Introduction

Artificial insemination (AI) of ewes with frozen-thawed ram semen often results in low (20% to 30%) conception rates [1]. Fresh diluted and chilled ram semen is an alternative to frozen storage [2]. Ram sperm motility was improved after storage at 4˚C for 2 to 3 days as compared with frozen storage for several weeks [2] [3]. Additionally, AI conception rate was higher when ram semen was stored at 4˚C or 23˚C for 1 to 3 days compared with several weeks of storage at −196˚C [2]. Conflicting results have been reported for the most suitable temperature for cold storage of ram semen, as some authors reported improved viability at 5˚C [4] while others reported better results after storage at 15˚C [5].

Milk or TRIS (tris[Hydroxymethyl]aminomethane) extenders are the most common extenders used for liquid storage of ram semen [6]. A TEST (N-Tris (hydroxymethyl)-methyl-2-aminoethane sulfonic acid), based extender has been evaluated in other species but not for the extension and liquid storage of ram semen [7] [8]. Choline based extender (CJ-2) was initially formulated to overcome harmful effects of sodium for cryopreservation of oocytes [9] [10]. Bull semen extended in CJ-2 egg yolk extender maintained acceptable motility for up to 7 days of storage at 4˚C [11]. There are no reports of using CJ-2 extender for extension and chilled storage of ram semen.

Inclusion of egg yolk in the extender protects ram spermatozoa during cold storage [12]. Milk and TRIS extenders with 5% egg yolk are commonly used for liquid storage of ram semen [6] [13]. The literature is void of studies on optimal percentage of egg yolk to maintain ram sperm motility during cool storage. Ethylene glycol (EG) has been used as a cryoprotectant of choice for cryopreservation of embryos and ram semen [14]. Ethylene glycol causes the least osmotic stress to spermatozoa during cryopreservation compared with other cryoprotectants [15] [16]. The addition of 1.6% EG in the extender medium to extend *Chinchilla lanigera* semen increased sperm motility after storage for 72 h at 4˚C [17]. However, the addition of EG to semen extender for cold storage of ram semen has yet to be tested.

Penicillamine, hypotaurine, and epinephrine (PHE) have different modes of action, but collectively enhance sperm motility, sperm capacitation, and acrosomal reaction [18]. Addition of PHE decreased the time interval to oocyte penetration by bovine sperm [19]. Culture of frozen-thawed bull semen in semen extender with PHE increased most sperm motility parameters compared with absence of PHE [20]. Currently, no studies which compared the effect of addition of PHE on sperm motility parameters after cool storage of ram semen.

Hence, the objective of the present study was to compare four different extenders (milk, TRIS, TEST or CJ-2), and two temperatures (4˚C or 15˚C) for extension and cool storage of ram semen for up to 72 hours. A subsequent study evaluated the effects of milk and TRIS extenders (best two extenders from first study) with or without supplementation with egg yolk (5% or 20%), EG and PHE on ram sperm motility parameters stored at 4˚C for 72 hours.

2. Materials and Methods

2.1. Animal Management and Semen Collection

The study was conducted at the University of Arkansas, Fayetteville, AR. All animal procedures were approved by Institutional Animal Care and Use Committee (Protocol # 15019). Clinically healthy mature Katahdin rams (n = 5) raised on mixed tall fescue (*Schedonorus arundinacea*) and bermudagrass (*Cynodon dactylon*) pasture were used in Experiment 1. In Experiment 2, Katahdin (n = 7) and Suffolk (n = 2) rams over 1 year of age were used. Katahdin rams were raised similar to those in Experiment 1, whereas Suffolk rams were maintained on mixed pasture containing white ladino clover (*Trifolium repens*) and bermudagrass (*Cynodon dactylon*). All rams had access to water, and mineral salt, and 0.5 kg/day of corn/soybean meal (17% crude protein, 82% total digestible nutrients on a dry matter basis) per ram. Semen was collected by electroejaculation into 15 ml polystyrene tubes, screw capped and placed immediately into a thermo-flask containing water maintained at 30˚C. Semen samples were transported to the laboratory for analysis.

2.2. Evaluation of Fresh Semen by Computer Assisted Sperm Analysis

All semen samples were evaluated within 2 hours of collection, using a computer assisted sperm analysis (CASA; Hamilton-Thorne, IVOS, Beverly MA) with version 12 TOX IVOS software. Sperm motility parameters were analyzed using the default settings recommended by manufacturer for ram semen. Semen samples were diluted in TRIS extender without egg yolk and placed in disposable 20 µm deep, 2-chambered IVOS slide (Cytonix, Beltsville, MD). A minimum of 400 spermatozoa was counted in each sample. Variables measured were motility (%), progressive motility (%), track speed (VCL, µm/second), path velocity (VAP, µm/second), progressive velocity (VSL, µm/second), beat frequency (BCF, MHz), lateral amplitude (ALH, µm), linearity (LIN, %), and straightness (STR, %).

2.3. Semen Extender Preparation—Experiment 1

All semen extender ingredients were purchased from Sigma Chemical (St. Louis, MO, USA) unless otherwise stated. Milk extender was prepared by dissolving 10 g of organic skim milk powder (NOW Real Food®, organic, non-fat dry milk powder), in 95 ml of purified water in a 250-ml beaker, heating to 95˚C on a hot plate and stirring constantly for 10 minutes. After cooling to room temperature, 0.5 g of fructose and 0.5 ml of gentamicin (10 mg/ml; Life Technologies, Gibco®) were added. The stock solution was stored at 4˚C until the day of semen collection, when 5 ml of egg yolk was added, mixed thoroughly and the extender warmed to 30˚C.

TEST extender was prepared following method as described previously [7]. Solutions of 7.5 g of TES and 3.93 g of TRIS were prepared in separate beakers

containing 100 ml purified water. The TES solution was titrated with TRIS to a pH of 7.2. Fructose (0.5 g) and 0.5 ml of gentamicin (Life Technologies, Gibco®) were added, the solution was filtered through a 0.2 µm filter and stored at 4˚C. On the day of semen collection, 5 ml of egg yolk was added to 95 ml of the stock solution as described for milk extender.

TRIS extender was prepared by adding 3.63 g of TRIS and 1.99 g of citric acid to 100 ml purified water [6]. The pH of the resulting solution was 7.0. After addition of fructose and gentamicin as previously described for TEST, the solution was then filtered through a 0.20 µm sterile filter and stored at 4˚C. Egg yolk was added on the day of use, as described for TEST extender.

Choline based extender was prepared according to the method described previously [10] with modification. Gentamicin was used instead of kanamycin sulfate. Lactic acid, hemi-calcium salt was substituted for calcium chloride dihydrate. Glucose was substituted with fructose, and phenol red was not added. The pH of the solution was 7.5. After addition of fructose and gentamicin as previously described for TEST, the solution was then filtered through a 0.20 µm sterile filter and stored at 4˚C. Egg yolk was added on the day of use, as described for TEST extender.

2.4. Processing and Storage of Semen—Experiment 1

Semen was initially extended to 600 million sperm/ml in each extender. Extended semen (50 µl aliquots) was then stored in 0.25 ml semen straws (IMV, Paillette Crista, France). The straws were initially filled with 50 µl of respective extender alone, separated by 50 µl volume of air, then 50 µl of experimental extended semen, another 50 µl volume of air, then 50 µl of extender to insulate the extended semen. Total of 24 straws were prepared from each ram semen collection. The straws were heat sealed and placed in flasks containing water at 30˚C. Twelve of the semen straws representing each extender and semen collection were placed in a refrigerator at 4˚C while the remaining straws were placed in a cooler (Koolatron PC3, Koolatron, Brantford, Ontario, Canada) maintained at 15˚C. Extended semen reached the desired storage temperatures within 2 to 3 hours of storage.

2.5. Semen Extender Preparation—Experiment 2

Milk and TRIS extenders were prepared as described for Experiment 1, but as 4× stock solutions. After determining the sperm concentration of each semen collection, the extenders were appropriately diluted to a 1× concentration so that extender ingredient concentrations were consistent across semen collections with varying sperm concentrations. After the proper semen dilution was calculated, purified water, egg yolk (5% or 20% v/v), and 0 or 1% ethylene glycol were added to each 4× extender to achieve a 1× solution. Then, the appropriate amount of semen to achieve 600 million sperm/ml was added to each extender combination across semen collections and maintained at 30˚C until storage.

A penicillamine, hypotaurine and epinephrine (PHE) stock solution was prepared as previously described [21]. The PHE stock solution was stored in 1.0 ml cryogenic vials at −20˚C until use on the day of use, when 40 µl of PHE stock was added to per ml of appropriate extended semen treatments. The final concentration in extenders was 20 µM penicillamine, 10 µM hypotaurine and 2 µM epinephrine.

2.6. Processing and Storage of Semen—Experiment 2

Each semen collection was distributed across extender treatments. Semen extender treatments consisted of either milk or TRIS based extenders, supplemented with either 5% or 20% egg yolk and the presence or absence of 1% EG. Extended semen samples were stored in sterile 1.5 ml Eppendorf tubes, placed in a beaker containing 300 ml of water at 30˚C and allowed to cool to 4˚C in a refrigerator over a 2 to 3 hour period. At each daily evaluation, sperm motility parameters were evaluated before and again after addition of PHE.

2.7. Evaluation of Extended Semen after Storage

Extended semen was evaluated after 24, 48, and 72 hours of storage in both experiment 1 and 2. An aliquot of 15 µl of chilled semen was diluted with 300 µl of pre-warmed TRIS extender (at 35˚C) in a 1.5 ml Eppendorf tube. Diluted semen samples were stained with 5 µl of 10 µg/ml Hoechst 33,342 solution before analysis, using the IVOS IDENT illumination option. Semen samples were maintained at 35˚C for 15 minutes before CASA, as described for fresh semen. In Experiment 2, semen samples were analyzed by CASA before and again after PHE addition.

2.8. Statistical Analysis

Data for sperm motility parameters measured at 24, 48 and 72 hours of storage were analyzed as repeated measures using MIXED procedure of SAS [22]. Variables in the model included extender, temperature, time (as a repeated measures of 24, 48, and 72 hours), and all 2- and 3-way interactions. Ram was considered a random effect and treatment (extender and temperature) considered a fixed effect. Non-significant ($P > 0.10$) interactions were removed from the model. The final model included extender, temperature × extender, and extender × hour interactions. Data are presented as least squares means ± standard error. Means separation occurred using PDIFF option of SAS. Dependent variables measured with IVOS were total motility, progressive motility, VCL, VAP, VSL, BCF, ALH, LIN, and STR.

3. Results

3.1. Experiment 1

Total motility of fresh semen was 48.4% ± 2.6%. Total motility decreased to 43.6% ± 2.4%, 31.1% ± 2.4%, and 27.2% ± 2.4% after extension and storage for

24, 48, and 72 hours, respectively ($P < 0.01$). Similarly, progressive motility of non-extended semen was 35.2 ± 2.3% and declined to 8.6% ± 0.7%, 5.3% ± 0.7%, and 3.2% ± 0.7% after extension and storage for 24, 48, and 72 hours, respectively ($P < 0.01$). Temperature × extender interaction ($P < 0.05$) was observed due to decreased total motility, VCL, VAP, BCF, ALH, and STR of sperm cells extended using CJ-2, and milk based extenders and stored at 15˚C (Table 1) compared with TRIS and TEST extenders at 15˚C. Semen extended using CJ-2 and stored at 15˚C exhibited temperature × extender interaction ($P < 0.05$) due to decreased progressive motility, and LIN compared with other extenders stored at 15˚C. A temperature × extender interaction ($P < 0.01$) was also observed mainly due to a decreased VSL of sperm cells stored in CJ-2, milk or TEST extenders and stored at 15˚C compared with semen extended using TRIS extender and stored at 15˚C. Progressive motility, VAP, and VSL of sperm cells stored at 4˚C using TRIS, CJ-2, and milk were similar ($P > 0.05$) but higher compared with TEST ($P < 0.05$). Lateral amplitude of sperm cells stored at 4˚C was higher in TEST or TRIS compared with CJ-2 and milk extenders ($P < 0.05$).

Table 1. Effect of temperature on ram sperm motility parameters, for semen extended using CJ-2, milk, TEST or TRIS extenders and stored at 4˚C or 15˚C for 72 hours.

Sperm motility parameter	Storage temperature	Extender treatment				
		CJ-2	Milk	TEST	TRIS	SE
Total motility	4˚C	$30.1^{b,x}$	$40.6^{ab,x}$	$30.4^{b,x}$	$44.5^{a,x}$	4.1
	15˚C	$9.1^{c,y}$	$20.1^{c,y}$	$41.3^{b,x}$	$55.2^{a,x}$	4.1
Progressive motility	4˚C	$6.8^{a,x}$	$7.8^{a,x}$	$2.8^{b,x}$	$7.4^{a,y}$	1.1
	15˚C	$1.0^{c,y}$	$4.9^{b,x}$	$3.8^{bc,x}$	$11.1^{a,x}$	1.1
Track speed	4˚C	$101.1^{a,x}$	$116.1^{a,x}$	$103.4^{a,x}$	$118.1^{a,x}$	6.9
	15˚C	$43.6^{c,y}$	$66.1^{b,y}$	$105.5^{a,x}$	$131.7^{a,x}$	6.9
Path velocity	4˚C	$56.3^{ab,x}$	$65.5^{a,x}$	$50.7^{b,x}$	$60.6^{ab,x}$	3.9
	15˚C	$22.9^{d,y}$	$38.3^{c,y}$	$51.9^{b,x}$	$66.2^{a,x}$	3.9
Progressive velocity	4˚C	$42.5^{a,x}$	$45.2^{a,x}$	$34.1^{b,x}$	$42.9^{a,x}$	2.9
	15˚C	$17.1^{c,y}$	$29.6^{b,y}$	$34.4^{b,x}$	$46.5^{a,x}$	2.9
Beat frequency	4˚C	$33.3^{a,x}$	$34.1^{a,x}$	$32.5^{a,x}$	$34.7^{a,x}$	2.5
	15˚C	$18.2^{b,y}$	$22.8^{b,y}$	$31.1^{a,x}$	$33.5^{a,x}$	2.5
Lateral amplitude	4˚C	$5.1^{b,x}$	$5.4^{ab,x}$	$6.6^{a,x}$	$6.6^{a,x}$	0.4
	15˚C	$2.8^{b,y}$	$3.6^{b,y}$	$6.4^{a,x}$	$6.7^{a,x}$	0.4
Linearity	4˚C	$39.2^{a,x}$	$40.3^{a,x}$	$35.1^{a,x}$	$36.8^{a,x}$	2.1
	15˚C	$18.4^{b,y}$	$29.7^{a,y}$	$33.8^{a,x}$	$34.7^{a,x}$	2.1
Straightness	4˚C	$65.6^{a,x}$	$67.2^{a,x}$	$65.1^{a,x}$	$67.1^{a,x}$	4.5
	15˚C	$32.5^{c,y}$	$48.3^{b,y}$	$63.7^{a,x}$	$63.6^{a,x}$	4.5

^{abcd}Values within the same row with unlike superscripts are different ($P < 0.05$). ^{xy}Values within the same column with unlike superscripts differ ($P < 0.05$).

Track speed, BCF, LIN, and STR of sperm cells stored at 4˚C was similar across extenders ($P > 0.05$).

There was extender × hour interaction ($P < 0.05$; Table 2), due to reduction of

Table 2. Effect of time of storage on ram sperm motility parameters, for semen extended using CJ-2, milk, TEST or TRIS and stored at 4˚C or 15˚C for 72 hours.

Semen extender	Time of evaluation, hours			
	24	48	72	SE
Track speed				
CJ-2	109.9^{a,x}	59.8^{b,z}	47.2^{b,z}	8.5
Milk	111.2^{a,x}	89.8^{a,y}	72.1^{b,y}	8.5
TEST	110.5^{a,x}	106.2^{a,xy}	96.8^{a,x}	8.5
TRIS	134.6^{a,x}	127.8^{a,x}	112.2^{a,x}	8.5
Path velocity				
CJ-2	60.9^{a,xy}	32.9^{b,z}	25.1^{b,z}	4.8
Milk	65.1^{a,xy}	51.1^{b,y}	39.7^{b,y}	4.8
TEST	53.7^{a,xy}	52.1^{a,y}	48.1^{a,x}	4.8
TRIS	67.5^{a,x}	65.6^{a,x}	57.1^{a,x}	4.8
Progressive velocity				
CJ-2	46.1^{a,x}	25.3^{b,z}	17.8^{b,yz}	3.5
Milk	49.4^{a,x}	37.1^{b,y}	25.6^{c,y}	3.5
TEST	35.4^{a,y}	34.7^{a,y}	32.8^{a,xy}	3.5
TRIS	47.1^{a,x}	47.3^{a,x}	39.7^{a,x}	3.5
Beat frequency				
CJ-2	38.1^{a,x}	22.8^{b,y}	16.4^{b,y}	3.6
Milk	34.4^{a,x}	27.9^{ab,xy}	23.1^{b,y}	3.6
TEST	32.1^{a,x}	31.5^{a,x}	31.8^{a,x}	3.6
TRIS	33.2^{a,x}	35.1^{a,x}	34.4^{a,x}	3.6
Linearity				
CJ-2	40.7^{a,xy}	25.9^{b,y}	19.9^{b,y}	3.4
Milk	44.7^{a,x}	33.5^{ab,xy}	26.9^{b,xy}	3.4
TEST	33.2^{a,y}	34.2^{a,xy}	36.6^{a,x}	3.4
TRIS	37.7^{a,y}	37.2^{a,x}	35.5^{a,x}	3.4
Straightness				
CJ-2	69.7^{a,x}	43.3^{b,z}	34.2^{b,z}	5.6
Milk	72.1^{a,x}	56.1^{b,xy}	45.5^{b,yz}	5.6
TEST	62.8^{a,x}	64.3^{a,x}	66.1^{a,x}	5.6
TRIS	62.2^{a,x}	68.5^{a,x}	65.4^{a,x}	5.6

^{abcd}Values within the same row with unlike superscripts differ ($P < 0.05$). ^{xyz}Values within the same column with unlike superscripts differ ($P < 0.05$).

VAP, VSL, BCF, LIN, and STR of sperm stored in CJ-2 and milk extenders at 48 and 72 hours of storage compared with that stored in TEST or TRIS extenders for the same period. A trend of extender × hour interaction ($P = 0.06$) was observed due to reduction of VCL of sperm cells in semen stored in CJ-2 and milk extenders at 48 and 72 hours of storage compared with semen stored in TRIS or TEST extenders and stored for same duration.

3.2. Experiment 2

Total motility of fresh sperm cells was 67 ± 1.8%. Total motility declined to 53.1% ± 1.6%, 46.2% ± 1.6%, and 40.7% ± 1.6% after extension and storage for 24, 48, and 72 hours, respectively ($P < 0.01$). Similarly, progressive motility of fresh sperm cells was 38.6% ± 1.3% and declined to 24.4% ± 0.9%, 20.1% ± 0.9%, and 17.1% ± 0.9% after extension and storage for 24, 48, and 72 hours respectively ($P < 0.01$). Total motility ($P = 0.001$), progressive motility ($P < 0.001$), VAP ($P = 0.001$), VSL ($P < 0.001$), BCF ($P = 0.002$), LIN ($P = 0.001$), and STR ($P = 0.07$) of sperm cells was higher or tended to be higher with milk compared with TRIS extender; however, ALH was higher in TRIS compared with milk ($P = 0.007$; Table 3). Supplementation of either 5% or 20% egg yolk resulted in similar total motility, progressive motility, VAP, VSL, BCF, LIN, and ALH ($P > 0.05$; Table 4). Supplementation of EG increased total motility ($P < 0.001$), progressive motility ($P < 0.001$) and BCF ($P = 0.03$) compared with no EG, STR tended to be higher ($P = 0.06$), but other parameters were similar (Table 5). Sperm total motility ($P = 0.004$), progressive motility ($P = 0.039$), VCL ($P = 0.036$), VAP ($P = 0.003$), VSL ($P = 0.001$), LIN ($P = 0.043$), and STR ($P = 0.044$) were reduced in extenders without EG and stored by 48 to 72 hours of storage (EG × hour, $P < 0.05$; Table 6). Addition of PHE before semen analysis increased sperm progressive motility ($P = 0.001$), and BCF ($P = 0.001$; Table 7), and tended to increase

Table 3. Sperm motility parameters after extension using either milk or TRIS and stored at 4˚C for 72 hours.

Sperm parameter	Extender			*P*-value
	Milk	TRIS	SE	
Total motility	49.7	43.6	1.3	0.001
Progressive motility	24.5	16.6	0.7	<0.001
Track speed	185.6	182.5	4.2	0.600
Path velocity	104.7	95.1	1.9	0.001
Progressive velocity	83.3	70.6	1.4	<0.001
Beat frequency	32.9	30.3	0.6	0.002
Lateral amplitude	6.8	7.4	0.2	0.007
Linearity	45.9	41.6	0.9	0.001
Straightness	75.5	72.5	1.2	0.070

Table 4. Sperm motility parameters after extension using 5% or 20% egg yolk in milk or TRIS and stored at 4˚C for 72 hours.

Sperm parameter	Egg yolk percentage			*P*-value
	5%	20%	SE	
Total motility	45.9	47.3	1.3	0.463
Progressive motility	20.3	20.7	0.7	0.721
Track speed	180.3	187.9	4.3	0.208
Path velocity	97.8	102.6	1.9	0.124
Progressive velocity	75.6	78.4	1.4	0.163
Beat frequency	31.4	31.8	0.6	0.641
Lateral amplitude	7.2	7.3	0.2	0.151
Linearity	42.9	44.6	0.9	0.185
Straightness	73.1	75.1	1.2	0.231

Table 5. Sperm motility parameters after extending with or without ethylene glycol in milk or TRIS.

Sperm parameter	Treatments			*P*-value
	Ethylene glycol	Control	SE	
Total motility	51.2	42.1	1.3	<0.001
Progressive motility	23.1	18.1	0.7	<0.001
Track speed	184.5	183.6	4.2	0.873
Path velocity	100.2	99.6	1.9	0.826
Progressive velocity	77.5	76.4	1.4	0.589
Beat frequency	32.5	30.6	0.6	0.029
Lateral amplitude	7.2	7.1	0.2	0.831
Linearity	44.3	43.1	0.9	0.345
Straightness	75.5	72.5	1.17	0.060

total motility ($P < 0.06$), and STR ($P = 0.08$) compared with no addition. However, PHE had no effect on VCL, VAP, VSL, and LIN of sperm cells ($P > 0.05$).

4. Discussion

Experiment 1, compared four different extenders (milk, TEST, TRIS, and CJ-2) at two storage temperature (4˚C or 15˚C) for extension and storage of ram semen using a similar concentration of egg yolk, fructose and gentamycin. Overall, ram semen stored at 4˚C was superior over storage at 15˚C, and the use of milk or TRIS based extender maintained better sperm motility parameters compared with CJ-2 extender at 4˚C. However, progressive motility, which is also a predictor of *in vivo* fertility, declined by 75% within 24 hours, and continued to decline over time regardless of the extenders used. Hence, in experiment 2, the aim was to determine whether additional egg yolk, and cryoprotectant at the time of

Table 6. Sperm motility parameters after extension with or without ethylene glycol in milk or TRIS with 5% or 20% egg yolk and stored at 4˚C for 72 hours.

Sperm parameter	Hours	Treatments		
		Ethylene glycol	Control	SE
Total motility	24	$55.4^{a,x}$	$50.9^{a,x}$	2.3
	48	$48.6^{a,y}$	$43.4^{a,y}$	2.3
	72	$49.6^{a,xy}$	$31.7^{b,z}$	2.3
Progressive motility	24	$26.1^{a,x}$	$23.1^{a,x}$	1.3
	48	$21.5^{a,y}$	$18.7^{a,y}$	1.3
	72	$21.5^{a,y}$	$12.6^{b,z}$	1.3
Track speed	24	$186^{a,x}$	$197^{a,x}$	7.4
	48	$182^{a,x}$	$191^{a,x}$	7.4
	72	$185^{a,x}$	$162^{b,y}$	7.4
Path velocity	24	$103.3^{a,x}$	$109.1^{a,x}$	3.3
	48	$98.4^{a,x}$	$104.5^{a,x}$	3.3
	72	$99.1^{a,x}$	$85.2^{b,y}$	3.3
Progressive velocity	24	$80.3^{a,x}$	$84.1^{a,x}$	2.4
	48	$75.7^{a,x}$	$80.2^{a,x}$	2.4
	72	$76.4^{a,x}$	$65.1^{b,y}$	2.4
Linearity	24	$46.5^{a,x}$	$47.2^{a,x}$	1.5
	48	$43.8^{a,x}$	$45.2^{a,x}$	1.5
	72	$42.8^{a,x}$	$37.1^{b,y}$	1.5
Straightness	24	$76.5^{a,x}$	$77.1^{a,x}$	2.1
	48	$75.1^{a,x}$	$74.6^{a,x}$	2.1
	72	$72.1^{a,x}$	$65.7^{b,y}$	2.1

^{ab}Values within the same row followed by different superscripts are different ($P < 0.05$). ^{xyz}Values within the same column with unlike superscripts differ ($P < 0.05$).

Table 7. Effect of addition of penicillamine, hypotaurine, and epinephrine (PHE) on sperm motility parameters before evaluation of cold stored ram semen, stored at 4˚C for 72 hours.

Sperm parameter	Treatments			*P*-value
	PHE	Control	SE	
Total motility	48.4	44.8	1.3	0.058
Progressive motility	22.4	18.7	0.7	0.001
Track speed	179.8	188.3	4.2	0.161
Path velocity	98.4	101.4	1.9	0.276
Progressive velocity	77.5	76.3	1.4	0.541
Beat frequency	32.9	30.2	0.6	0.001
Lateral amplitude	7.1	7.4	0.2	0.152
Linearity	44.5	43.1	0.9	0.267
Straightness	75.5	72.5	1.1	0.078

extension using milk or TRIS, and supplementation of PHE before evaluation of sperm cells might maintain or improve progressive motility. Results indicated that milk extender supplemented with 1% EG, 20% egg yolk, and addition of PHE before analysis is capable of maintaining progressive motility of ram semen at approximately 60% of its initial value when stored at 4˚C for up to 72 hours.

Seminal plasma maintains semen pH, but semen pH may fluctuate after extension [23]. In order to maintain sperm cell viability or avoid damage to cells in extended semen and storage, pH needs to be constant [23]. Buffers are used to minimize pH fluctuation after extension. Ideal buffers for semen extension should have temperature-independent buffering capacity, be neutral to cells, and does not react with other components of the extender [24] [25]. Milk, TRIS, TES, and CJ-2 extenders meet most of the above criteria to be used as an ideal buffer in semen extenders [1] [10] [25].

In the current study, milk or TRIS based extenders maintained better motility parameters compared with TEST or CJ-2 extenders. However, there was no difference in most motility parameters using either milk or TRIS extenders when stored at 4˚C using similar concentrations of egg yolk, citric acid, fructose, and gentamycin. Results from the current study are consistent with some earlier findings [26] but contrary to results reported by others [3] [27] [28] [29] [30]. The conception rate after vaginal insemination of ewes with fresh semen extended with milk containing 5% egg yolk was greater when compared with fresh semen extender with TRIS containing 20% egg yolk [31]. In a preliminary trial, 19 Gulf Coast Native ewes were artificially inseminated during September with fresh semen extended in milk or TRIS containing 5% egg yolk (unpublished observations). The conception rate was 50% (5/10) for ewes in the milk extender group and 56% (5/9) for ewes in the TRIS extender group. Further study with larger ewe numbers are needed for comparison of these extenders and supplements.

At 15˚C, most sperm motility parameters were similar for up to 24 hours of storage. However, after that sperm motility parameters declined more rapidly in milk or CJ-2 extenders compared with TEST or TRIS extenders. Such decreases were not apparent after storage at 4˚C. In general, ram sperm membrane damage occurs during cooling from 30˚C to 5˚C, accompanied by a rapid decrease in progressive motility and percentage of intact acrosomes, and such effects are less likely when cooled from 30˚C to 15˚C [32]. It is because phase transition of membrane lipids occurs when spermatozoa are cooled to 5˚C, causing loss of selective permeability and leading to damage in phospholipid bilayer in the plasma membrane [33]. However, in the current study, it is not known why a greater reduction of motility of sperm cells was observed when stored using milk or CJ-2 extenders at 15˚C. Choline is a component of the phospholipid bilayer and helps in stabilizing cell plasma membrane for protection of spermatozoa and oocytes. In other studies, choline based extenders provided better medium for storage of oocytes compared with traditional sodium containing medium [9] [10] [34]. Bull

semen extended and cold-stored at 4˚C for 7 days using CJ-2 extender had equal or higher sperm motility parameters compared with semen stored in synthetic caudal epididymal plasma [11].

Milk contains lipids, lactose, casein, albumin, globulin, lactoferrins, and several other compounds. Heating milk to 95˚C for 10 minutes inactivates lactenin in protein, a compound toxic to sperm cells [6]. Several studies were done in the past to find component(s) in milk that protect sperm cells after extension and storage of semen, ruling out individual components such as lipid fraction [35], lactose, or fructose [36]. However, casein proteins (not albumin or globulin) increased bull sperm motility after extension and storage [36]. Binder of sperm (BSP) proteins is present in seminal plasma that deteriorates sperm cells after extension [37]. In ram semen, casein micelles bound to BSP proteins decreases cholesterol and phospholipid loss from the sperm membrane, thus protecting sperm cells after extension [38]. Nevertheless, this property of casein in milk extender failed to protect sperm cells after extension and storage at 15˚C.

During the process of cooling extended semen for liquid storage, massive rearrangements and leakage of sperm plasma membrane occurs which could lead to sperm cell death [39]. Inclusion of egg yolk in the culture medium protects ram spermatozoa from cell death [12]. It is still unclear which components of egg yolk and the mechanism for protection of ram sperm cells. Inclusion of TRIS + 20% egg yolk better protected sperm cells against cold shock compared with inclusion of TRIS + 15% egg yolk, and TRIS + 10% egg yolk [40]. TRIS or sodium citrate extender with 20% egg yolk can maintain better sperm motility compared with milk extender containing 5.5% egg yolk [41]. However, the study was confounded as different extender components and different egg yolk percentages were used [41]. In the current study, milk or TRIS extenders were compared using either 5% or 20% egg yolk in extender medium. There was no difference in most motility parameters using 5% or 20% (v/v) of egg yolk in either extender. However, inclusion of 20% egg yolk in milk extender led to increased VCL and VAP compared with other combinations.

Ethylene glycol is a cryoprotectant of choice for freezing embryos because of its low molecular weight, which enables rapid influx into cells during equilibration and rapid efflux after thawing [14]. Similar effects of EG occurs across the plasma membrane of sperm cells [42]. Ethylene glycol at 2.5% concentration in TEST extender effectively protected ram semen stored for 3 hours at 4˚C compared with DMSO, propylene glycol, dimethylacetamide, and glycerol at 2.5%, 5%, and 10% concentrations [43]. Inclusion of 1.6% EG in TEST extender was beneficial for extension and storage of *Chinchilla lanigera* semen at 4˚C for 72 hours [17]. In the current study, addition of 1% EG in the extender for extension and storage increased most sperm motility parameters compared with no EG addition. In a preliminary trial, conception rate was compared using fresh or cool stored ram semen in an extender with EG during August in Midwestern US (unpublished observation). Semen was extended using milk extender (with 20%

egg yolk and 1% EG) and ewes inseminated immediately after extension (n = 27) or after extended semen was stored at 4˚C for up to 36 hours before insemination (n = 28). The conception rate in ewes inseminated using stored semen was 31% while fresh extension and insemination resulted in 37% conception rate.

Penicillamine, hypotaurine, and epinephrine have different modes of action but collectively enhance sperm motility, sperm capacitation, and acrosomal reaction [18]. Addition of PHE in the medium decreased time interval for oocyte penetration by sperm [19]. Frozen thawed and cultured bull semen in PHE-added medium increased most sperm motility parameters compared with no PHE [20]. Studies reporting the effects of PHE on sperm motility parameters of extended ram semen are rare. In the current study, addition of PHE before semen analysis increased progressive motility and BCF of sperm cells and tended to increase total motility and STR. In a separate preliminary trial, comparison was done to find the effect of addition or no addition of PHE (40 µl PHE/ml extender) in milk (20% egg yolk supplemented with 1% EG) extender before AI (unpublished observation). A total of 27 ewes were artificially inseminated with extended and cold stored ram semen for 12 to 24 hours at 4˚C. The PHE was added in extended semen 5 minutes before AI in PHE group (n = 14) or no PHE addition in control group (n = 13). The conception rate in the PHE added group was 50% while only 23% conceived in the no PHE group. Further studies are needed to confirm beneficial effect of addition of PHE in extended semen before AI.

5. Conclusions

In the first experiment, semen collected from each ram was divided into four aliquots and extended using either milk, TRIS, TEST, or CJ-2 and stored at 4˚C or 15˚C. Ram semen stored using milk or TRIS based extenders at 4˚C maintained similar motility parameters but better compared with TEST or CJ-2 extenders after storage for 72 hours. At 15˚C, most of the sperm motility parameters declined at 24 hours after storage using CJ-2 and milk extenders. Considering sperm progressive motility, which is also a predictor of *in vivo* fertility, milk and TRIS extenders stored at 4˚C had similar but higher progressive motility compared with ram semen extended in CJ-2 and TEST extenders. However, progressive motility of ram sperm declined by 75% when stored at 4˚C for 24 hours, and continued to decline over time regardless of extenders used.

In the second experiment, semen collected from each ram was distributed across treatment combination consisting of TRIS or milk extenders supplemented with 5% or 20% (v/v) egg yolk, and 0 or 1% EG and stored for 72 hours at 4˚C. Of the extenders examined, milk supplemented with 1% EG, 20% egg yolk before storage and addition of PHE after storage but before analysis was capable of maintaining progressive motility of ram semen at approximately 60% of its initial value when stored at 4˚C for 72 hours.

In a preliminary trial, conception rate was compared using fresh diluted or

cold stored for up to 36 hours using milk extender (with 20% egg yolk and 1% EG) for inseminating Katahdin ewes in Midwestern US during August. The conception rate in ewes was similar; however, further study is needed using large animal number for confirmation of this result.

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Conflicts of Interest

The authors declare no conflict of interest regarding this publication.

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