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Bovine Sperm Motility as Affected by Alpha Tocopherol and Ascorbic Acid during Storage

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Abstract

Our purpose was to determine the impact of ascorbic acid and α-tocopherol on bovine sperm motility following short-term storage or cryopreservation. Semen was collected from mature Angus bulls (n = 4). The experimental design was a randomized complete block, with bull serving as block, and treatments were structured as a 4 × 4 factorial with four concentrations of ascorbic acid (0, 5, 10, 20 mM) and four concentrations of α-tocopherol (0, 0.05, 0.5, 5 mM). Sperm motility characteristics were evaluated using computer assisted sperm analyses at 0, 4, and 8 h of incubation (39˚C) and post-cryopreservation. Initial sperm motility was (79.6% ± 1.6) and decreased to (6.1% ± 1.6%) after cryopreservation. Cryopreserved spermatozoa had lower ($P < 0.05$) post-thaw qualities when compared with fresh collections and for spermatozoa that had been incubated for 4 h at 39˚C. In contrast, most of the motility characteristics of spermatozoa that were incubated for 8 h at 39˚C were similar to those of post-thaw spermatozoa. Sperm motility, hyperactivity, and velocity characteristics were not affected ($P > 0.1$) by α-tocopherol. Addition of 20 mM ascorbic acid to storage media decreased ($P < 0.05$) sperm velocity traits, but the addition of 5 or 10 mM ascorbic acid did not alter sperm velocity. Sperm cell oxidation following cryopreservation/post-thaw was affected by an interaction ($P < 0.05$) between concentrations of ascorbic acid and α-tocopherol. Stepwise regression models predicted ($P < 0.05$) post-thaw motility and velocity characteristics of cryopreserved spermatozoa. Our results suggest that adding ascorbic acid and α-tocopherol was not beneficial for short-term storage of spermatozoa; however, our results were inconclusive with regards to inclusion of ascorbic acid and α-tocopherol in egg yolk-based cryopreservation media.

Keywords

Bull Spermatozoa, Antioxidants, Cryopreservation

1. Introduction

Spermatozoa navigation of the female reproductive tract and penetration of the ovum is essential for normal fertilization. Semen processing techniques have been associated with decreased sperm viability, induced cellular damage, and declines in conception rates [1] [2]. Bilodeau *et al.* [3] reported that cryopreservation depletes spermatozoa of their natural antioxidant supply, which results in increased cellular damage. Under those circumstances, free radical production is increased resulting in heightened oxidative stress [4] [5]. Identification of storage methods that do not impair sperm function and fertility is needed.

Antioxidants scavenge and quench free radicals [6] [7] [8]. Prevention of oxidative stress by supplementation of culture media and cryopreservation extenders with antioxidants has been investigated previously [9] [10] [11]. In more recent studies, lycopene decreased lipid peroxidation of spermatozoa and increased sperm viability and motility [12]. Via antioxidant mechanisms, leptin, carnitine, and lycopene reduced DNA damage during storage of spermatozoa [12] [13] [14]. Natural antioxidants, such as ascorbic acid and α-tocopherol, have produced conflicting results on sperm function post-preservation [7] [8] [15] [16], suggesting there may be an optimal threshold for antioxidant supplementation, and above that threshold, the beneficial effects may not be realized. Our objective was to determine the impact of ascorbic acid and α-tocopherol on bovine sperm motility following short-term storage or cryopreservation.

2. Materials and Methods

2.1. Semen Collection and Preparation

For 60 days prior to semen collection, retired mature-breeding bulls (Angus; n = 4) were maintained in a dry lot where they had free access to mixed grass hay, water, and mineral supplement. On the morning of sample days, semen was collected from each bull via electro-ejaculation (Electroejac IV; Neogen Corporation, Lansing, MI, USA) and placed in 15 mL conical centrifuge tubes. Ejaculates were transported to the lab in a 39˚C water bath. In a warm room (~32˚C) at the lab, ejaculates were centrifuged at 750 ×g for 10 min to remove seminal plasma. Spermatozoa were re-suspended in sperm TALP (mSPTL) [17], split into separate tubes with equal volumes, and washed once with mSPTL via centrifugation. Following that wash, one-half of each ejaculate was re-suspended in mSPTL for short-term storage at 39˚C without CO_2, and the other one-half was re-suspended in fraction A of the cryopreservation extender. Spermatozoa [25×10^6 in mSPTL (1 mL) containing antioxidant treatments] were incubated in a 39˚C water bath in 5 mL screw top tubes and evaluated at 4 and 8 h of storage. Spermatozoa used for cryopreservation were placed in fraction A (1 mL) and cooled to 5˚C. Both mSPTL and media for cryopreservation were prepared fresh each day.

2.2. Experimental Treatments

Antioxidants (ascorbic acid and α-tocopherol) and media reagents were pur-

chased from Sigma Aldrich (Saint Louis, MO, USA). Experimental treatments were structured as a 4 × 4 factorial with four concentrations of ascorbic acid (0, 5, 10, 20 mM) and four concentrations of *α*-tocopherol (0, 0.05, 0.5, 5 mM). Sperm motility characteristics were assessed after 0, 4, and 8 h of incubation (at 39˚C without CO_2) with antioxidant treatments and immediately after thawing for cryopreserved spermatozoa.

All sperm characteristics (motility, velocity, and size) were determined using computer assisted sperm analyses (CASA; Hamiliton Thorne IVOS, Beverly, MA). Spermatozoa were placed on a warm slide and motility assessed by Animal Motility Software, version 12.1. Sperm motility properties were defined as: Motile (% of total sperm moving at a track velocity ≥30 μm/sec and progressive velocity ≥15 μm/sec), Progressive (% of total sperm moving at a track velocity ≥50 μm/sec and straightness ≥70%), Rapid (Progressive % with path velocity >50 μm/sec), Medium (Progressive % with path velocity <50 μm/sec but >30 μm/sec), Slow (% of total sperm moving at a path velocity <30 μm/sec and progressive velocity <15 μm/sec), Static (% of spermotozoa with no movement at all), Path Velocity [VAP; average velocity of the smoothed cell path (μm/sec)], Progressive Velocity [VAP; average velocity measured in a straight line from the beginning to the end of the track (μm/sec)], Track Speed [VCL; average velocity measured over the point-to-point track (μm/sec)], Lateral Amplitude [ALH; Mean width (μm) of the head oscillation as the sperm swims], Beat Frequency (BCF; frequency of sperm head crossing the sperm average path in either direction), Straightness [STR; measures departure of average sperm path from straight line (ratio of VSL/VAP)], Linearity [LIN; measures departure of actual sperm path from straight line (ratio of VSL/VCL)], Elongation [ELONG; ratio of head width to head length (%)], and Sperm Head Size (AREA; average size in square microns of all sperm heads). Sperm hyperactivation status was defined based on Pfeifer *et al.* [18]; Non-hyperactivated (LIN > 53%, ALH < 7.2 μm, and VCL < 164.3 μm/s), Hyperactivated (LIN < 53%, ALH > 7.17 μm, and VCL > 164.3 μm/s), and samples that did not fit those two categories were considered in a Transitional Phase.

2.3. Cryopreservation

Fraction A semen extender consisted of sterile stock solution [TRIS Base (200 mM), citric acid monohydrate (6.6 mM), and D-fructose (55.5 mM)], and egg yolk (20%). Fraction B semen extender consisted of heat treated organic milk with glycerol (14%). Both fractions of semen extender were prepared to have a pH of 6.56 and an osmolarity of 290 mOsm. All antioxidant treatments were imposed in each ejaculate. Spermatozoa (2×10^8 in 1 mL) were placed in 5 mL screw top tubes containing fraction A and antioxidant treatments. Experimental tubes were stored in a cool room until their temperature reached 5˚C. Fraction B (5˚C), containing antioxidant treatments, was added stepwise every 10 min [10% (100 μL), 20% (200 μL), 30% (300 μL), 40% (400 μL)] until the final volume of extender reached 2 mL (1 ml fraction A, 1 mL fraction B). Samples equilibrated

for 3 h, after which, extended spermatozoa (50×10^6/0.5 mL) were loaded into 0.5 mL straws and placed in a rack to prepare for cryopreservation. The heat sealed straws were lowered into liquid nitrogen vapor at −15˚C/min (2.54 cm/min) starting at −10˚C, held for 10 min at −100˚C, and then plunged into liquid nitrogen for storage until evaluation at a later date.

2.4. Lipid Peroxidation Assay

Following motility assessment of cryopreserved spermatozoa, lipid peroxidation was determined using thiobarbituric acid reactive substances kit (TBARS Assay Kit, Cayman Chemical Company, Ann Arbor, MI, USA). Cryopreserved semen was thawed in a water bath (39˚C), diluted in warm phosphate-buffered saline (PBS; 1 mL), and centrifuged at 1000 ×g for 5 min in a warm room (32˚C). Following centrifugation, cryopreservation extender was removed and spermatozoa were re-suspended in PBS. Samples, containing 25×10^6 sperm cells, were sonicated in ice utilizing a Vibra Cell (Sonics and Materials Inc., Baton Rouge, LA, USA) three times for 5 sec intervals at 40 V setting. Samples were boiled for 1 h and then placed in ice for 10 min. Samples were then centrifuged for 10 min at 1600 ×g at 4˚C. Duplicates of each sample (150 µl) were loaded into a clear 96-well plate and malondialdehyde (MDA) concentrations were determined by absorbance (530 nm).

2.5. Statistical Analysis

Sperm motility characteristics were analyzed using mixed model procedures. Experimental design was a randomized complete block with bull serving as the block. Experimental unit was each well in a 96-well plate with antioxidant treatment, and time was the repeated measure. Antioxidant treatments were structured as a 4 × 4 factorial with four concentrations of ascorbic acid (0, 5, 10, 20 mM) and four concentrations of α-tocopherol (0, 0.05, 0.5, 5 mM). When F-tests were significant ($P < 0.05$), means were separated using multiple t-tests with Tukey's adjustment. Chi-square statistic was used to determine effect of treatments on proportion of hyper-activated spermatozoa. Using motility characteristics of fresh semen collections as predictor variables, stepwise multiple regression with forward selection was used to predict motility characteristics of bovine spermatozoa after cryopreservation.

3. Results

Storage method affected ($P < 0.05$) nearly all CASA motility characteristics (Table 1). For most sperm motility and velocity traits, cryopreserved spermatozoa had lower ($P < 0.05$) post-thaw qualities when compared with fresh collections and for spermatozoa that had been incubated for 4 h at 39˚C in vitro. In contrast, most of the motility characteristics of spermatozoa that were incubated for 8 h at 39˚C in vitro were similar to those of post-thaw spermatozoa (Table 1). Hyperactivity status of spermatozoa was affected ($P < 0.0001$) by storage

Table 1. Bovine sperm motility characteristics as affected by storage method.

Sperm	Storage Method[1]					
	Incubation time, h					
Characteristic[2]	Fresh	4	8	Cryo	SEM	P-value
Motility, %	79.6[a]	32.3[b]	8.1[c]	6.1[c]	1.86	0.0001
Progressive, %	63.3[a]	26.5[b]	3.8[c]	3.0[c]	1.64	0.0001
Rapid, %	76.0[a]	29.4[b]	5.1[c]	3.8[c]	1.75	0.0001
Static, %	10.8[b]	19.3[b]	38.2[a]	41.5[a]	3.73	0.0001
VAP, μm/s	120.0[a]	80.3[b]	67.4[c]	75.4[b]	1.88	0.0001
VSL, μm/s	99.4[a]	69.6[b]	56.7[c]	62.2[c]	1.83	0.0001
VCL, μm/s	199.3[a]	123.5[b]	106.1[c]	122.9[c]	3.06	0.0001
ALH, μm	6.8[a]	5.6[b]	5.4[b]	7.1[a]	0.15	0.0001
BCF, Hz	35.8[a]	30.7[b]	26.5[c]	24.7[c]	0.74	0.0001
STR, %	83.3[a]	82.7[ab]	81.1[ab]	81.1[b]	0.6	0.015
LIN, %	53.3	55.7	53.3	54.2	0.71	0.06
ELONG, %	43.3	43.5	41.3	44.2	0.7	0.047
AREA, μm^2	5.9	6.2	5.7	6.5	0.23	0.116

[a,b,c]within sperm motility characteristic, least-squares means without a common superscript differ ($P < 0.05$). [1]Storage Method categories were: Fresh (sperm motility characteristics were determined within 1 h of collection); 4 and 8 (spermatozoa were incubated at 39˚C in mSPTL before motility characteristics determined); and Cryo (spermatozoa were cryopreserved and sperm motility characteristics were determined post-thaw). [2]sperm motility was evaluated using computer assisted sperm analysis (see text for definitions).

method (Table 2). Spermatozoa from fresh semen samples were primarily (75%) in a Transitional Phase of hyperactivity and 0% Non-hyperactivated; whereas, after cryopreservation the spermatozoa were primarily (73%) in a Transitional Phase of hyperactivity but 23% of evaluated spermatozoa were Non-hyperactivated. Incubation for 4 or 8 h at 39˚C in vitro resulted in a lower proportion of Transitional Phase spermatozoa (Table 2).

Sperm motility, hyperactivity, and velocity characteristics were not affected ($P > 0.1$) by α-tocopherol. Sperm motility characteristics were numerically decreased as concentration of ascorbic acid increased. Addition of 20 mM ascorbic acid to storage media decreased ($P < 0.05$) sperm velocity traits; although numerically lower the addition of 5 or 10 mM ascorbic acid did not alter sperm velocity (Table 3). The percentage of spermatozoa with a Transitional Phase hyperactivity status after 4 h at 39˚C in vitro decreased ($P < 0.05$) with increasing concentrations of ascorbic acid (81%, 63%, 50%, and 31%; respectively for 0, 5, 10, and 20 mM ascorbic acid). For cryopreserved spermatozoa, Transitional Phase hyperactivity status increased ($P < 0.05$) with the addition of ascorbic acid (63% vs. 77%).

Sperm cell oxidation following cryopreservation/post-thaw was affected by an interaction ($P < 0.05$) between concentrations of ascorbic acid and α-tocopherol (Table 4). Malondialdehyde concentrations were highest for spermatozoa treated with the combination of 5 mM ascorbic acid and 0.05 mM α-tocopherol.

Table 2. Proportion of bovine sperm hyper-activation status as affected by storage method.

Sperm	Storage Method[1]			
	Incubation Time, h			
Motility Pattern[2]	Fresh[a]	4[b]	8[b]	Cryo[c]
Non-hyperactivated, %	0	44	48	23
Transitional Phase, %	75	56	50	73
Hyperactivated, %	25	0	2	3

[a,b,c]proportion of spermatozoa exhibiting patterns of hyper-activation was affected ($P < 0.0001$) by storage method; proportions without a common superscript differ ($P < 0.05$). [1]Storage Method categories were: Fresh (sperm motility characteristics were determined within 1 h of collection); 4 and 8 (spermatozoa were incubated at 39˚C in mSPTL before motility characteristics determined); and Cryo (spermatozoa were cryopreserved and sperm motility characteristics were determined post-thaw). [2]Sperm Motility Pattern was defined as Non-hyperactivated (LIN > 53%, ALH < 7.2 μm, and VCL < 164.3 μm/s), Hyperactivated (LIN < 53%, ALH > 7.17 μm, and VCL > 164.3 μm/s), and samples that did not fit those two categories were considered in a Transitional Phase.

Table 3. Effects of ascorbic acid on bovine sperm motility characteristics.

Sperm	Ascorbic Acid, mM				SEM	P-value
Characteristic[2]	0	5	10	20		
Motility, %	35.9	32.9	32.7	30.7	1.83	0.26
Progressive, %	26.6	24.9	23.0	22.0	1.57	0.18
Rapid, %	31.7	29.2	27.4	26.0	1.73	0.12
Static, %	27.8	23.1	26.6	32.3	3.69	0.38
VAP, μm/s	91.4[a]	87.5[ab]	84.0[bc]	80.3[c]	1.86	0.0007
VSL, μm/s	75.7[a]	74.1[ab]	70.6[ab]	67.6[b]	1.82	0.012
VCL, μm/s	147.9[a]	137.7[ab]	135.3[b]	131.0[b]	3.04	0.002
ALH, μm	6.4[a]	6.4[ab]	6.3[ab]	5.8[b]	0.15	0.029
BCF, Hz	31.2[a]	29.3[ab]	28.9[ab]	28.2[b]	0.73	0.037
STR, %	81.1	82.8	81.8	82.4	0.59	0.18
LIN, %	53.2[b]	56.0[a]	53.5[ab]	53.7[ab]	0.73	0.037
ELONG, %	44.0	43.2	42.5	42.5	0.69	0.37
AREA, μm^2	5.8	5.9	6.2	6.5	0.23	0.19

[a,b,c]within sperm motility characteristic, least-squares means without a common superscript differ ($P < 0.05$). [1]Storage Method categories were: Fresh (sperm motility characteristics were determined within 1 h of collection); 4 and 8 (spermatozoa were incubated at 39˚C in mSPTL for 4 or 8 hours; respectively, before motility characteristics determined); and Cryo (spermatozoa were cryopreserved and sperm motility characteristics were determined post-thaw). [2]sperm motility was evaluated using computer assisted sperm analysis (see text for definitions).

Whereas, the malondialdehyde concentrations were lowest for all treatments containing 20 mM ascorbic acid, or those treatments containing no ascorbic acid with 0.05 or 0.5 mM α-tocopherol (Table 4). Stepwise regression models predicted ($P < 0.05$) post-thaw motility and velocity characteristics of cryopreserved spermatozoa (Table 5). Sperm velocity traits, specifically VAP, of fresh semen collections were most frequently selected for prediction of post-thaw characteristics of bovine spermatozoa.

Table 4. Interactive effects of ascorbic acid and *α*-tocopherol on cryopreserved bovine sperm oxidation[1].

Ascobic	*α*-Tocopherol, mM			
Acid, mM	0	0.05	0.5	5
0	4.8[ab]	2.0[b]	2.2[b]	4.0[ab]
5	3.8[ab]	5.7[a]	4.2[ab]	3.0[ab]
10	3.2[ab]	2.8[ab]	3.5[ab]	3.1[ab]
20	2.1[b]	2.3[b]	2.2[b]	2.0[b]

[a,b]least-squares means (SEM = 0.654) without a common superscript differ ($P < 0.05$). [1]main effects of ascorbic acid ($P = 0.0009$) and *α*-tocopherol ($P = 0.83$) were not presented based on main effects interaction ($P = 0.025$).

Table 5. Motility characteristics of fresh bovine spermatozoa as predictors of motility of spermatozoa after cryopreservation.

Characteristic[1]	Stepwise Regression Equation	R-square	*P*-value
Motility, %	81.26 + 0.15 (LIN) − 14.15 (AREA)	0.99	0.021
Progressive, %	−73.38 + 1.68 (MED) + 0.59 (VAP)	0.99	0.038
Rapid, %	−60.22 + 1.54 (MED) + 0.49 (VAP)	0.99	0.075
VAP, μm/s	−28.38 + 0.21 (Progressive) + 2.15 (ELONG)	0.99	0.033
VSL, μm/s	−154.34 + 2.4 (VAP) − 1.4 (LIN)	0.99	0.021
VCL, μm/s	−739.85 + 4.86 (VCL) − 14.81 (ALH)	0.99	0.01

[1]stepwise multiple regression, using forward selection, was used to predict motility characteristics of bovine spermatozoa after cryopreservation based on motility characteristics of fresh semen collections. Fresh predictor variables were: Motility, Progressive, Rapid, Medium, Slow, Static, VAP, VSL, VCL, ALH, BCF, STR, LIN, ELONG, and AREA (see text for definitions).

4. Discussion

Semen processing techniques can reduce the natural antioxidant supply normally found in semen [3] [19]. Our project focused on antioxidant effects and storage method on sperm motility characteristics and lipid oxidation. We found that addition of 5 or 10 mM ascorbic acid to preservation media for bovine spermatozoa was neither beneficial nor detrimental to sperm motility; however, addition of 20 mM ascorbic acid decreased sperm velocity. In contrast, Beconi *et al.* [11] demonstrated that addition of 5 mM of ascorbic acid improved bovine sperm motility. We found no benefit of adding *α*-tocopherol to preservation media. Previous research indicated that including *α*-tocopherol (0.1 mM and 1.0 mM) in preservation media was beneficial to ram spermatozoa [16], and bovine spermatozoa benefited from the addition of *α*-tocopherol (2 mM) [8]. Both of those results are contradictory to our findings, but there are no obvious explanations except for species and evaluation methods.

There is evidence that ascorbic acid can serve both as a pro-oxidant and an antioxidant [20] [21]. In our study, pro-oxidant effects were observed for those spermatozoa cryopreserved with a combination of ascorbic acid (5 mM) and

α-tocopherol (0.05 mM). Ascorbic acid did not have significant antioxidant properties until inclusion rates were 20 mM. It is intriguing that the same concentrations of ascorbic acid decreased sperm motility characteristics and prevented lipid oxidation. Ascorbic acid acted as a scavenger and reduced free radicals, but the net result still leads to reduced sperm motility during extended storage methods. This implies that free radical production is not the only issue that impacts spermatozoa during storage.

Previous studies have shown that higher quality semen benefits more from antioxidant supplementation than lower quality semen [11]. We evaluated initial motility differences directly after ejaculation, but we did not perform other initial evaluations that are linked to sperm viability. Future projects may benefit from evaluating other parameters linked to sperm viability immediately after ejaculation. Cryopreservation negatively impacts factors such as DNA fragmentation, mitochondrial membrane potential, and acrosomal integrity, which have been linked to sperm viability [22].

Pregnancy rates can be impacted by many factors including use of estrous synchronization, intensity of estrus, use of sexed semen, and percentage of motile spermatozoa [23]. In our study, bull served as a blocking factor for our experiment and proved to be a significant source of variation (data not shown), which is consistent with previous reports [23]. As males mature, it can be difficult to predict the ability of spermatozoa to fertilize ova in vivo; that prediction is complicated by many factors including bull breed and sperm characteristics [23] [24]. Pregnancy rates have been associated with sperm hyperactivity [18], and sperm velocity [25]. Our prediction analyses found that velocity characteristics of fresh semen were the most informative predictors of sperm quality following cryopreservation; specifically, VAP was selected in half of the traits predicted. Additional research is needed, but VAP was found to be the most useful sperm characteristic relative to pregnancy rates and fertility of bulls [26]. Although sperm cryopreservation and ART are already commonly used throughout the world, finding ways to eliminate stressful conditions for spermatozoa during storage could significantly benefit the entire cattle industry.

5. Conclusion

Storage methods compromise the capacity for fertilization of spermatozoa. Supplementation of sperm cryopreservation extender with ascorbic acid can reduce lipid peroxidation of sperm membranes; however, it concomitantly reduced sperm motility and velocity characteristics. In contrast, *α*-tocopherol had no significant effects on sperm motility, but did decrease sperm membrane lipid peroxidation. Future research will focus on understanding the mechanisms and linkages among sperm velocity characteristics, reactive oxygen species, and fecundity.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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