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Abstract
Cyclodextrins improve post-thaw viability and motility of semen as well as mediate cholesterol efflux and subsequent acrosome reaction in spermatozoa from several species. The objectives of this study were: (a) to assess the effect of prefreeze addition of 60 mM hydroxypropyl-β-cyclodextrin (β-CD) on post-thaw viability and motility of jack and stallion semen cryopreserved in ethylene glycol-based freezing extenders containing 5% or 20% (v/v) egg yolk (LEY and HEY, respectively), and (b) to evaluate the ability of 1 μM calcium ionophore A23187 and/or 60 mM β-CD to induce acrosome reaction in thawed jack and stallion spermatozoa. Post-thaw motility of spermatozoa cryopreserved in HEY was higher ($P < .05$) for jack but lower ($P < .05$) for stallion spermatozoa when compared with LEY. Jack and stallion spermatozoa both exhibited higher ($P < .05$) motility when cryopreserved in 60 mM β-CD than without β-CD. Curvilinear velocity was faster ($P < .05$) for jack and stallion spermatozoa cryopreserved in LEY than in HEY. A treatment × time interaction affected ($P < .05$) the proportion of spermatozoa that underwent acrosome reaction. Post-thaw incubation of jack and stallion spermatozoa with β-CD for 90 minutes induced acrosome reaction in 85% and 22% of viable sperm cells, respectively; however, only 32% of jack and 8% of stallion spermatozoa incubated with calcium ionophore underwent acrosome reaction. This study is the first to evaluate the effect of β-CD (not loaded with cholesterol) on jack semen cryopreservation, and results reveal that β-CD may be a useful tool to enhance semen cryopreservation and to induce post-thaw acrosome reaction in jack spermatozoa.

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The Effect of 2-Hydroxypropyl-β-Cyclodextrin on Post-Thaw Parameters of Cryopreserved Jack and Stallion Semen

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ABSTRACT

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1. Introduction

Barker and Gandier reported the first equine pregnancy and live birth from the use of frozen-thawed stallion semen in 1957 [1]. Polge and Minotakis [2] reported acceptable post-thaw motility of cryopreserved jack semen in 1964, and in 1967 Krause and Grove [3] reported the first successful pregnancy and live birth of a mule foal resulting from use of frozen-thawed jack semen. Improvements in the techniques for cryopreservation and artificial insemination have led to an increase in the use of frozen-thawed semen in equine species. However, frequency of use of cryopreserved jack semen is less than that of stallion semen [4].

In several regions of the world, jacks are mated with mares to produce mule foals. Advantages associated with the use of frozen-thawed jack semen for that purpose are numerous and include: (1) eliminate intimidation of mares by the sexual behavior of jacks during courtship and mating, (2) overcome physical limitations of a small jack servicing large breed mares, and (3) easier movement of genetic resources as semen versus live animals. Use of frozen-thawed jack semen may also be helpful to preserve

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and propagate endangered donkey species such as the Poitou jackass [5-7].

Sperm cryopreservation and capacitation studies investigating cyclodextrins have been conducted in a number of species. Cyclodextrins are water-soluble macrocyclic oligosaccharides that contain a hydrophobic pocket that can accommodate nonpolar substances such as cholesterol [8-10]. Cyclodextrins have been complexed with cholesterol and used as a source of supplemental cholesterol for cryopreserved spermatozoa of several species [11-17]. Supplemental cholesterol in this form has improved post-thaw motility and viability but inhibits post-thaw acrosome reaction and fertility of equine and bovine semen [12,15-18]. Various media have been supplemented with unbound cyclodextrin (not complexed with cholesterol) to induce cholesterol efflux, which has led to sperm capacitation in humans and other species [8,16,19-25]. In addition, unbound cyclodextrin has been added to semen extender before freezing and has improved post-thaw parameters of cryopreserved boar semen [26-29].

The objectives of this study were: (a) to assess the effect of prefreeze addition of 60 mM hydroxypropyl-β-cyclodextrin (β-CD) (unbound) on post-thaw viability and motility of jack and stallion semen cryopreserved in ethylene glycol-based freezing extenders containing low (5% v/v) or high (20% v/v) concentrations of egg yolk (which contains cholesterol), and (b) to evaluate the ability of calcium ionophore A23187 (CaI) and/or β-CD (unbound) to induce acrosome reaction in thawed jack and stallion spermatozoa in vitro.

2. Materials and Methods

2.1. Animals

2.1.1. Jacks

Three healthy jacks of proven fertility were used for this project. The client-owned jacks, ranging from 5 to 13 years of age and from 273 to 432 kg in body weight, were housed at the Iowa State University College of Veterinary Medicine Teaching Hospital during the semen collection period.

2.1.2. Stallions

Five healthy stallions of proven fertility, ranging from 8 to 18 years of age and from 454 to 591 kg in body weight, were used for this study. The stallions (two Quarter horses, two American Paint horses, and one Thoroughbred) were either client-owned or owned by the Iowa State University Department of Animal Science. All stallions were housed at Iowa State University during the semen collection period. One stallion had previously established pregnancies through insemination with frozen-thawed semen, but the other four had not been tested previously in that capacity.

2.2. Semen Collection

In the 10-day period immediately before the start of the study, two ejaculates were collected from each male to facilitate partial emptying of epididymal sperm reserves.

Semen was collected from jacks with the use of a sedated, ovariectomized, and hormonally stimulated mount mare. Each ejaculate was collected using a Missouri-type artificial vagina and was taken to the laboratory immediately for processing.

All stallions were trained to serve a phantom, and semen was collected using methods similar to that for jacks.

For the study, ejaculates were collected four times from each stallion and each jack, yielding 20 stallion and 12 jack ejaculates.

2.3. Semen Processing and Analysis

On arrival at the laboratory, the gel fraction of each ejaculate was physically removed with a nonspermicidal syringe before filtration with quick filtration material (Miracloth; Calbiochem, LaJolla, CA) into a prewarmed graduated cylinder. Immediately after recording of the gel-free ejaculate volume, motility was visually assessed. All semen samples had greater than 60% motility and were further examined for their suitability for cryopreservation. Sperm cell concentration was determined through the use of a Bright-Line hemocytometer (American Optical, Buffalo, NY), and morphology of spermatozoa was assessed after eosin/nigrosin staining [30]. The 20 stallion ejaculates averaged 67 $\pm$ 5.3 mL volume, 75% $\pm$ 2.3% motility, and 74% $\pm$ 3.8% normal morphology. The 12 jack ejaculates averaged 76 $\pm$ 10.3 mL volume, 80% $\pm$ 1.9% motility, and 90% $\pm$ 1.9% normal morphology.

After initial semen analysis was completed, raw samples were extended with EZ Mixin OF (Animal Reproduction Systems, Chino, CA) to a concentration of 50×10^6 spermatozoa/mL of extended semen. Extended semen from each ejaculate was split into four aliquots (one for each experimental freezing extender; —see later in the text) and placed in prewarmed 50-mL conical centrifuge tubes. Tubes were subsequently placed into a 35°C water bath, and the bath was immediately placed in a refrigerator (5°C) to cool.

2.4. Freezing Extender Treatments

The base freezing extender consisted of EZ Mixin OF containing 2% (v/v) ethylene glycol. We chose ethylene glycol as the cryoprotectant because glycerol moderately inhibits fertilization in the stallion [31] and also because glycerol can be more toxic than ethylene glycol for cryopreserved stallion semen and cooled jack semen [32,33]. Treatments included low (5% v/v) or high (20% v/v) egg yolk (LEY and HEY, respectively) and 0 or 60 mM 2-β-CD (Sigma-Aldrich Company, St. Louis, MO; molecular weight (MW) = 1,576) in a 2 $\times$ 2 factorial treatment arrangement.

Egg yolk was prepared by using an egg separator to remove the egg white. The intact egg yolk was rolled on sterile filter paper to absorb additional egg white before piercing the membrane and collecting the yolk in a semen aspiration cone (IMV Technologies, Cat. No. 005533). We chose to use unbound β-CD instead of cholesterol-loaded cyclodextrin as a means to bind cholesterol present in the egg yolk. Further, we chose a 60 mM concentration of β-CD because previous work in our laboratory [34] produced a 58.5% mare pregnancy rate with jack semen cryopreserved in 60 mM β-CD.

2.5. Cryopreservation Process

Once the extended semen had cooled to 5°C (2-3 hours), it was centrifuged in a refrigerated centrifuge at 400 × *g* for 10 minutes. The supernatant was siphoned off and discarded. The remaining sperm cell pellet was resuspended in the designated experimental freezing extender to a final concentration of 400×10^6 spermatozoa/mL. Samples were then placed in 0.5-mL polyvinylchloride straws. Total equilibration time in the freezing extender, including the time necessary to hand fill the straws, was 30 minutes. Straws were placed on stainless steel racks, placed in liquid nitrogen vapor (−160°C) for a minimum of 20 minutes, packed into canes, and then plunged into liquid nitrogen where they were stored until thawing.

2.6. Semen Thawing

Each straw of semen was removed from the liquid nitrogen dewar and immediately placed in a 37°C water bath for 30 seconds.

2.7. Post-Thaw Semen Analysis

2.7.1. Computer-Assisted Sperm Analysis

Motility characteristics of spermatozoa were analyzed after thawing using computer-assisted sperm analysis (CASA; IVOS Sperm Analyzer, version12.1p; Hamilton-Thorne Research, Inc., Beverly, MA). Parameter settings for the IVOS were as follows: frames acquired = 45, minimum contrast = 70, minimum cell size = 4 pixels, threshold straightness (STR) = 75%, medium average path velocity (VAP) cutoff = 50 μm/sec, low VAP cutoff = 20 μm/sec, nonmotile head size = 6 pixels, nonmotile head intensity = 106, static intensity limits = 0.68-1.40, and static elongation = 12-97.

Thawed samples were diluted to a final concentration of 20×10^6 spermatozoa/mL with prewarmed (37°C) modified Kenney's extender (VP-BB-USA904 equine breeding buffer, IMV International Corp., Maple Grove, MN) to circumvent difficulties performing CASA in a high egg yolk environment. Samples were maintained for 5 minutes at 37°C, and 2 μL of diluted semen were placed into one end of a disposable 20-μm-deep chamber (Leja 4 Chamber [Hamilton Thorne Biosciences, Beverly, MA]). The percentage of motile sperm as well as standard CASA parameters, average path velocity (VAP) straight line (progressive) velocity (VSL), curvilinear velocity (VCL), lateral head displacement (ALH), cross-beat frequency (BCF), straightness (STR), linearity (LIN) were recorded.

2.7.2. Induction of Acrosome Reaction

One hundred μl of thawed semen were placed in a 0.5-mL microcentrifuge tube. Samples were treated with 0 or 60 mM β-CD and 0 or 1 μM CaI in a modified 2 × 2 factorial treatment arrangement (semen treated with β-CD before freeze was not exposed to additional β-CD after thaw). Treated samples were maintained at 37°C to enable aliquots to be removed for staining at 0, 60, and 90 minutes after treatment. We chose to investigate the induction of acrosome reaction because it has not yet been fully investigated in the jack and also because of its potential relationship to fertility in the field or in the laboratory when performing in vitro fertilization.

Plasma membrane and acrosomal membrane integrity were assessed after exposure to CaI and/or β-CD using a triple-stain flow cytometric procedure [35]. The final concentrations of stains were 12 μM propidium iodide, 100 nM SYBR 14, and 2.5 μg/mL peanut agglutinin conjugated with phycoerythrin.

Staining of semen samples occurred in an incubator (5% carbon dioxide in humidified air at 37°C) for 15 minutes, and stained spermatozoa were analyzed with a Beckman Coulter XL-MCL flow cytometer (Beckman Coulter, Inc., Indianapolis, IN). The machine was set on low flow rate, and excitation of stains was achieved with a 488-nm argon laser. Fluorescence detector 1 (505-545 nm) detected the viable cells (staining green with SYBR-14), whereas fluorescence detector 2 (560-590 nm) detected the acrosome-reacted cells (staining orange with peanut agglutinin conjugated with phycoerythrin). Fluorescence detector 3 (605-635 nm) detected the dead cells (staining red with propidium iodide). A total of 20,000 cell events per sample was counted.

2.8. Statistical Analysis

All data analyses were done within species. The effect of prefreeze treatment (egg yolk, β-CD) on post-thaw semen parameters (determined through CASA) was assessed by analysis of variance procedures using the PROC GLIMMIX procedure in SAS Software (SAS Institute, Inc. of Cary, NC) [36]. Similarly, PROC GLIMMIX was used in a split-plot analysis to examine the effect of post-thaw treatment with CaI and/or β-CD on the in vitro induction of acrosome reaction. Mean separations were performed for all pairwise comparisons calculating degrees of freedom independently for each comparison (Satterthwaite method, $\alpha = 0.05$), and data are reported as least squares means ± standard error of the mean.

3. Results

3.1. Effect of Prefreezing Treatment of Spermatozoa

3.1.1. Jacks

Individual jack affected ($P < .01$) post-thaw motility of spermatozoa, which ranged from 17% to 25%. Egg yolk and β-CD concentration also affected post-thaw motility. Post-thaw motility was higher ($P < .01$) in HEY than in LEY (23% ± 0.7% and 20% ± 0.7%, respectively) and higher ($P < .01$) in 60 mM than in 0 mM (23% ± 0.7% and 20% ± 0.7%, respectively) β-CD. No interaction ($P < .49$) between egg yolk and β-CD was observed. Motility in each treatment combination was as follows: LEY = 18% ± 1.0%, LEY + β-CD = 21% ± 1.0%, HEY = 22% ± 1.0%, HEY + β-CD = 24% ± 1.0%.

Egg yolk concentration affected VAP, VSL, VCL, STR, and LIN. VAP, VSL, and VCL were faster ($P < .01$) for spermatozoa cryopreserved in LEY (92 ± 5.8, 82 ± 5.4, and 151 ± 8.0 μm/sec, respectively) than in HEY (79 ± 5.8, 68 ± 5.4, and 131 ± 8.0, respectively). STR and LIN were higher ($P < .01$ and $P < .03$, respectively) for sperm frozen in LEY (82% ± 1.2% and

Table 1
Least square means (±SEM) for post-thaw parameters of jack semen as measured by CASA

Freeze Treatment	Motility (%)	VAP (μm/sec)	VSL (μm/sec)	VCL (μm/sec)	ALH (μm)	BCF (Hz)	STR (%)	LIN (%)
LEY	18 (3.3)[b]	94 (5.9)[a]	84 (5.5)[a]	152 (8.1)[a]	6.0 (0.27)[b]	42 (1.1)[a]	83 (1.3)[a]	53 (0.8)[a]
LEY + β-CD	21 (3.3)[a,b]	90 (5.9)[a]	80 (5.5)[a]	150 (8.1)[a]	6.1 (0.27)[b]	42 (1.1)[a]	82 (1.3)[a,b]	51 (0.8)[a,b]
HEY	22 (3.3)[a]	80 (5.9)[b]	70 (5.6)[b]	133 (8.1)[b]	6.1 (0.28)[b]	38 (1.1)[b]	80 (1.3)[b]	51 (0.8)[a,b]
HEY + β-CD	24 (3.3)[a]	77 (5.9)[b]	65 (5.6)[b]	128 (8.1)[b]	6.8 (0.28)[a]	35 (1.1)[c]	77 (1.3)[c]	50 (0.8)[b]

VAP, average path velocity; VSL, straight line (progressive) velocity; VCL, curvilinear velocity; ALH, lateral head displacement; BCF, cross-beat frequency; STR, straightness; LIN, linearity; CASA, computer-assisted sperm analysis; SEM, standard error of the mean.
[a,b,c]Means within a column with unlike superscripts are different ($P < .05$).

52% ± 0.7%, respectively) than in HEY (79% ± 1.2% and 50% ± 0.7%, respectively).

An interaction between egg yolk concentration and β-CD concentration was observed for ALH ($P <. 05$) and BCF ($P < .02$). Results are presented in Table 1.

3.1.2. Stallions

Individual stallion affected ($P < .01$) post-thaw motility of spermatozoa, which ranged from 12% to 24%. Egg yolk and β-CD concentration also affected post-thaw motility. Post-thaw motility was higher ($P < .05$) in LEY than in HEY (17% ± 0.4% and 15% ± 0.4%, respectively) and higher ($P < .01$) in 60 mM than in 0 mM β-CD (17% ± 0.4% and 15% ± 0.4%, respectively). No interaction ($P < .64$) between egg yolk and β-CD was observed. Post-thaw motility in each treatment combination was as follows: LEY = 16% ± 0.6%, LEY + β-CD = 17% ± 0.6%, HEY = 14% ± 0.6%, HEY + β-CD = 17% ± 0.6%.

Additional post-thaw parameters of stallion semen measured by CASA are shown in Table 2. Egg yolk concentration affected VCL, ALH, BCF, and LIN. VCL was faster ($P < .01$) for spermatozoa cryopreserved in LEY (124 ± 4.5 μm/sec) than in HEY (117 ± 4.5 μm/sec). ALH was less ($P < .01$) for cells frozen in LEY (6.6 ± 0.22 μm) than in HEY (7.2 ± 0.22 μm), and BCF was higher ($P < .01$) for sperm cryopreserved in LEY (38 ± 0.6 Hz) than in HEY (34 ± 0.6 Hz). LIN was lower ($P < .01$) for spermatozoa frozen in LEY (44% ± 1.1%) than in HEY (47 ± 1.1).

Treatment with β-CD affected VCL and tended to affect VAP and VSL. VCL was faster ($P < .01$) for spermatozoa cryopreserved in 0 mM (123 ± 4.5 μm/sec) than in 60 mM (118 ± 4.5 μm/sec) β-CD. VAP and VSL tended ($P < .09$ and $P < .08$, respectively) to be faster in spermatozoa frozen in 0 mM (69 ± 3.0 and 56 ± 3.1 μm/sec, respectively) than in 60 mM (67 ± 3.0 and 54 ± 3.1 μm/sec, respectively) β-CD.

3.2. Effect of Post-Thaw Treatment to Induce Acrosome Reaction

3.2.1. Jacks

The proportion of viable sperm cells that underwent acrosome reaction was affected ($P < .01$) by a treatment × time interaction. Spermatozoa treated after thaw with 60 mM β-CD, alone or in combination with 1 μM CaI, had the highest proportion of acrosome reaction at 90 minutes (85% and 82%, respectively), whereas spermatozoa treated only with CaI were not different than controls at 90 minutes after treatment (Table 3).

3.2.2. Stallions

The proportion of viable sperm cells that underwent acrosome reaction was affected ($P < .01$) by a treatment × time interaction. Spermatozoa treated after thaw with β-CD had the highest proportion of acrosome reaction at 90 minutes (22%), and treatment with CaI, alone or in combination with β-CD, resulted in an increased percentage of acrosome-reacted sperm cells (8% and 15%, respectively) compared with controls at 90 minutes after treatment (Table 4).

4. Discussion

Various forms of β-CDs have been directly added to freezing extenders for cryopreservation of boar semen. Zeng and Terada [27-29] showed improvement in post-thaw acrosome integrity and motility parameters of boar semen when either 2-hydroxypropyl-β-cyclodextrin or methyl-β-cyclodextrin (neither bound with cholesterol) was added to the freezing extender during a 3-hour cooling process. Other researchers [26] also observed an increase in post-thaw viability of boar semen cryopreserved with unbound 2-hydroxypropyl-β-cyclodextrin at a concentration of 40 or 60 mM. Those authors suggested cyclodextrin acted as a cholesterol shuttle to increase the sperm plasma membrane cholesterol:phospholipid ratio, reducing cold shock sensitivity of the spermatozoa. Our findings are similar to those previous studies that showed a beneficial effect of unbound β-CD when used in a HEY extender.

Post-thaw motility of jack spermatozoa frozen in HEY + β-CD was higher than that of spermatozoa cryopreserved in LEY in the present study, and post-thaw motility of stallion sperm frozen in β-CD was also higher, irrespective of egg yolk concentration. Although post-thaw motility of

Table 2
Least square means (±SEM) for post-thaw parameters of stallion semen as measured by CASA

Freeze Treatment	Motility (%)	VAP (μm/sec)	VSL (μm/sec)	VCL (μm/sec)	ALH (μm)	BCF (Hz)	STR (%)	LIN (%)
LEY	16 (2.5)[a,b]	69 (3.1)[a]	56 (3.2)[a]	127 (4.7)[a]	6.6 (0.25)[b]	38 (0.6)[a]	74 (1.2)[a]	44 (1.2)[b]
LEY + β-CD	17 (2.5)[a]	67 (3.1)[a]	54 (3.2)[a]	121 (4.6)[a,b]	6.6 (0.24)[b]	38 (0.6)[a]	74 (1.2)[a]	44 (1.2)[b]
HEY	14 (2.5)[b]	69 (3.1)[a]	56 (3.2)[a]	119 (4.7)[b]	7.1 (0.25)[a,b]	35 (0.6)[b]	75 (1.2)[a]	47 (1.2)[a]
HEY + β-CD	17 (2.5)[a]	67 (3.1)[a]	54 (3.2)[a]	115 (4.6)[b]	7.3 (0.24)[a]	34 (0.6)[b]	73 (1.2)[a]	46 (1.2)[a]

VAP, average path velocity; VSL, straight line (progressive) velocity; VCL, curvilinear velocity; ALH, lateral head displacement; BCF, cross-beat frequency; STR, straightness; LIN, linearity.
[a,b]Means within a column with unlike superscripts are different ($P < .05$).

Table 3
Proportion of jack sperm cells that underwent acrosome reaction as measured by a triple-stain procedure

Species	Post-Thaw Treatment*	Observation Time (minutes)	Percentage of Acrosome-Reacted Cells (±SEM)**
Jack	β-CD	0	12 (2.1)[f]
		60	74 (2.1)[b]
		90	85 (2.1)[a]
	β-CD + CaI	0	13 (2.1)[f]
		60	72 (2.1)[b]
		90	82 (2.1)[a]
	CaI	0	13 (1.5)[f]
		60	28 (1.5)[d,e]
		90	32 (1.5)[c]
	C	0	11 (1.5)[f]
		60	25 (1.5)[e]
		90	31 (1.5)[c,d]

* C, control (no post-thaw treatment) (n = 144 samples); CaI, 1 μM calcium ionophore A23187 (n = 144 samples); β-CD, 60 mM β-CD (n = 72 samples); β-CD + CaI, 1 μM calcium ionophore A23187 and 60 mM β-CD (n = 72 samples).

** Expressed as a proportion of spermatozoa that were viable after thaw.

[a,b,c,d,e,f]Means within a column with unlike superscripts are different ($P < .05$).

spermatozoa for any of the freezing treatments in either species fell short of the industry goal of more than or equal to 30% minimum post-thaw motility (such as the 31.5% motility reported by Brazilian researchers [37]), our results exceeded the 15% minimum standard used to determine freezability in one study [38]. Despite the relatively low post-thaw motility in the present study, it should be noted that our laboratory obtained pregnancy rates exceeding 58% with jack semen cryopreserved in a similar manner and possessing similar post-thaw motility [34], thus indicating that in vitro assessment of post-thaw motility is not always predictive of in vivo fertility. Our results are also comparable with the 23% progressive motility observed in "low viability" donkeys in one study [39].

All ejaculates in this study were centrifuged at 5°C instead of room temperature (22°C) to standardize the handling of samples owing to the slightly longer time involved with transport of the stallion ejaculates to the laboratory (during which slight cooling may have occurred). Vidament et al. [40] observed reduced post-thaw motility of stallion semen when centrifugation occurred at 4°C rather than 22°C, and we speculate that centrifugation at 5°C may have also adversely affected our results. In addition, one cannot overlook the fact that individual male responses to cryopreservation [41] could have contributed to the somewhat lower post-thaw motility observed in our study.

Table 4
Proportion of stallion sperm cells that underwent acrosome reaction as measured by a triple-stain procedure

Species	Post-Thaw Treatment*	Observation Time (minutes)	Percentage of Acrosome-Reacted Cells (±SEM)**
Stallion	β-CD	0	4 (1.0)[e,f]
		60	14 (1.0)[b]
		90	22 (1.0)[a]
	β-CD + CaI	0	4 (1.0)[e,f]
		60	10 (1.0)[c]
		90	15 (1.0)[b]
	CaI	0	3 (0.6)[f]
		60	6 (0.6)[d]
		90	8 (0.6)[c]
	C	0	3 (0.6)[f]
		60	4 (0.6)[e,f]
		90	5 (0.6)[d,e]

* C, control (no post-thaw treatment) (n = 240 samples); CaI, 1 μM calcium ionophore A23187 (n = 240 samples); β-CD = 60 mM β-CD (n = 120 samples); β-CD + CaI, 1 μM calcium ionophore A23187 and 60 mM β-CD (n = 120 samples).

** Expressed as a proportion of spermatozoa that were viable after thaw.

[a,b,c,d,e,f]Means within a column with unlike superscripts are different ($P < .05$).

Post-thaw viability of stallion spermatozoa did not vary over time (0, 60, and 90 minutes after thaw), but viability of jack spermatozoa exhibited a decrease of nearly 25% of viable cells. This result agrees with a previous report [42] that showed that donkey spermatozoa are more sensitive than stallion spermatozoa to low temperatures. Our finding also supports the idea of performing direct thaw insemination with frozen-thawed jack semen as a means to potentially avoid this decline in motility [34]. Further investigation into the reason for the decline in post-thaw viability of jack semen is clearly warranted.

Post-thaw treatment of jack semen with 60 mM β-CD caused 85% of viable sperm cells to undergo the acrosome reaction, 74% within 60 minutes. Although stallion sperm cells also responded favorably to post-thaw treatment with β-CD (a 6-fold increase in acrosome reaction from 0 to 90 minutes), they did so to a much lesser extent (only 22% underwent acrosome reaction at 90 minutes). Perhaps a longer duration post-thaw stallion semen treatment would increase the proportion of sperm cells undergoing the acrosome reaction; however, our result with stallion semen is similar to the 14%-17% cyclodextrin-induced acrosome reaction reported by Spizziri et al. [43].

Although CaI is commonly used to induce sperm acrosome reaction, it was less effective than β-CD at inducing acrosome reaction in both stallions and jacks at the concentration used in this study (1 μM). Other researchers have used concentrations ranging from 70 nM [44] to 5 [45] μM or 10 μM [46] to induce acrosome reaction in stallions, and perhaps the proportion of acrosome-reacted spermatozoa would have been if we had used a concentration higher than 1 μM.

Other researchers have demonstrated that the addition of cyclodextrin in vitro mediates cholesterol efflux from the sperm membrane, thus inducing capacitation and acrosome reaction in spermatozoa from humans, mice, goats, stallions, and bulls [8,16,19-24]. Belmonte et al. [8] showed that addition of 1 mM cyclodextrin to human sperm in vitro facilitated normal membrane fusion processes and cholesterol efflux as opposed to nonspecific mechanisms. Cross [9] reported the addition of methyl-β-cyclodextrin to human sperm caused a dose-dependent loss of up to 89% of membrane cholesterol in 30 minutes, compared with ~30% loss in 24 hours in a medium devoid of methyl-β-cyclodextrin. An increase in sperm binding to zona pellucida and an increase in spontaneous acrosome reaction have been reported for human ejaculates exposed to 2-hydroxypropyl- β-cyclodextrin [22]. The addition of β-CD to mouse and bovine spermatozoa in vitro induced tyrosine phosphorylation, capacitation, and acrosome reaction in the absence of BSA [25], and this same

phenomenon also was demonstrated by Osheroff et al. with human spermatozoa [46]. Presumably, stallion and jack semen undergo the acrosome reaction by a mechanism similar to that described in other species when β-CD is added to in vitro to sperm cells.

Ethylene glycol (2% v/v) was used as the cryoprotectant for this project. Ethylene glycol has been shown to be a suitable replacement for glycerol for cryopreservation of stallion semen by various research groups [32,47,48]. Glycerol is deleterious to the fertility of fresh or cryopreserved jack semen [33,49], and this fertility loss was not observed when dimethylformamide or ethylene glycol replaced glycerol in fresh extended jack semen. Researchers in France have investigated the use ethylene glycol for cooled (4°C) and frozen donkey semen [49], but to our knowledge, our current study and our previous study [34] are the only others to document successful use of ethylene glycol for cryopreservation of jack semen.

In summary, we demonstrated that post-thaw motility of jack semen was improved with the prefreeze addition of β-CD to a high egg yolk freezing extender containing 2% (v/v) ethylene glycol. In addition, we showed that β-CD is a powerful sperm acrosome reaction inducing agent for jack spermatozoa. These results, the first reported for jack spermatozoa treated with unbound β-CD and evaluated after thaw, give encouragement for further study, which may lead to enhancement of artificial insemination of equids using frozen-thawed semen. We also documented that β-CD may be used to induce acrosome reaction, which is a noteworthy finding because it is a safer alternative (poses fewer human health hazards) than CaI. Further studies are required, however, to elucidate the specific biological mechanisms through which prefreezing treatment with β-CD enhances post-thaw motility and through which post-thaw treatment with β-CD enhances sperm acrosome reaction for jacks.

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