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Semen cryopreservation in the Indian rhinoceros (Rhinoceros unicornis)

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Abstract

The objective was to identify an extender and cryoprotectant combination for Indian rhinoceros (*Rhinoceros unicornis*) sperm that yielded high post-thaw sperm quality. Male Indian rhinoceroses ($n = 6$; 7.5–34 yr old) were anesthetized and subjected to a regimented electroejaculation procedure (75–100 mAmps; 4–10 volts; 7–150 stimuli; total of 10 electroejaculation procedures). High quality semen fractions from each ejaculate were divided into four aliquots and a 2 x 2 factorial design used to compare the effect of two sperm extenders (standard equine [EQ] and skim milk-egg-yolk-sugar [SMEY]), and two cryoprotectants (glycerol and dimethylsulfoxide [DMSO]). Cyropreserved samples were thawed and assessed for motility, viability and acrosome integrity over time. Electroejaculate fractions processed for cryopreservation had high sperm concentration (516×10^6/mL) and motility (79%). Post-thaw sperm characteristics were higher ($P < 0.05$) when semen was cryopreserved in EQ versus SMEY. Post-thaw motility of sperm cyropreserved in EQ averaged 50–55% compared to 22–37% in SMEY, with no significant differences in sperm characteristics of samples cyropreserved in glycerol and DMSO. In conclusion, sperm collected from Indian rhinoceroses via electroejaculation were cryopreserved using EQ extender with either glycerol or DMSO; post-thaw quality was adequate for use in assisted reproductive procedures.

Keywords: Indian rhinoceros; Sperm cryopreservation; Spermatozoa; Semen; Testosterone

1. Introduction

Recent advances in the development of artificial insemination (AI) in the Indian rhinoceros (*Rhinoceros unicornis*) resulted in the first successful pregnancy using frozen-thawed sperm [1]. Subsequent research demonstrated a pregnancy could also be established in the African white rhino (*Ceratotherium simum simum*) through AI of frozen-thawed sperm [2]. Comprehensive semen cryopreservation studies have been conducted in the African black (*Diceros bicornis michaeli, D.b minor*) [3,4], African white (*C.s. simum, C.s. cottoni*) [4–9], and Sumatran rhinoceros (*Dicerorhinus sumatrensis*) [3]. However, comparative research with Indian rhinoceros sperm is lacking.

Semen cryopreservation in conjunction with genome resource banking could provide a valuable means of preserving the genetic life of individual Indian rhinoceroses. The captive Indian rhinoceros population exhibits an unequal distribution of founder genetics [10]. Founder Indian rhinoceroses captured

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from a highly inbred population [11,12] have contributed to 97% of the captive population [13]. In addition, three founder individuals were responsible for ~50% of the genes in all captive-born Indian rhinoceroses [13]. Natural breeding attempts in the Indian rhinoceros frequently result in severe aggression between the male and female; this behavioral incompatibility has made genetic management of the species a challenge. Within the last 5 yr, development of AI has become a priority for the Indian rhinoceros Species Survival Plan® (SSP) to facilitate the infusion of genes from under- or un-represented founders and individuals that descend from them. With an AI technique recently developed for this species [1], the next step is to optimize semen cryopreservation and integrate genome resource banking and AI into the management strategy to improve the genetic health of the captive population.

With numbers of pregnancies established through AI of frozen thawed sperm in the rhinoceros very limited [1,2], factors affecting the success of this technique are still under evaluation. However, many factors influence the success of AI with frozen-thawed sperm in the horse, the closest domestic relative of the rhinoceros. Stallion variability is a major factor, but semen processing and evaluation techniques, in addition to sperm cryopreservation and thawing protocols, all have a role [14]. To date, only standard equine (EQ) cryoextender has been tested in Indian rhinoceros AI procedures using frozen-thawed sperm [1]. Egg yolk and/or milk based cyroextenders have been used to successfully freeze sperm from many species [15]; some cryoextenders containing skim milk proteins appeared to be more beneficial in protecting stallion sperm during cooling and freezing [16–20]. The cryoprotectants glycerol and dimethylsulfoxide (DSMO) have been used in conjunction with EQ to freeze Indian rhinoceros sperm that subsequently was used for AI, resulting in pregnancies [1]. In addition, African white rhinoceros (C. *s. simum*) sperm cryopreserved using DMSO [8,9] achieved pregnancy via AI [2].

The objective of the present study was to test two semen extenders (EQ and skim milk-egg yolk-sugar [SMEY]) and two cryoprotectants (glycerol and DMSO) for their ability to maintain pre-freeze sperm characteristics (motility, viability, acrosome status, and morphology) over time after thawing. In addition, this study assessed the reproductive status of genetically valuable male Indian rhinoceroses in the North American captive population.

2. Materials and methods

2.1. Animals

Six male Indian rhinoceroses located at four institutions in the USA participated in the study: Studbook Number (SB No.) 147, Cincinnati Zoo and Botanical Garden, Cincinnati, OH; SB No. 087 and 239, the Wilds, Cumberland, OH; SB No. 222 and 053, the Wildlife Conservation Society, Bronx, NY; SB No. 049, White Oak Conservation Center, Yulee, FL. Males ranged in age from 7.5–34 yr old; two were of proven fertility at the time of collection. Data were collected between November 2003 and December 2005.

2.2. Anesthesia and blood collection

Male rhinoceroses were fasted 24 h prior to anesthesia for electroejaculation. Water was withdrawn the morning of the procedure. Rhinoceroses were immobilized using a combination of 3.5–3.8 mg etorphine (M99, Wildlife Pharmaceuticals, Fort Collins, CO USA), 14–20 mg detomidine (Dormosedan, Pfizer, Exton, PA, USA), and 200–400 mg ketamine (Fort Dodge Laboratories, Inc., Fort Dodge, IA, USA) administered IM as a cocktail by projectile dart (Tel-Inject USA, INC., Agua Dulce, CA USA) or pole syringe (Dan-Inject Jabstick, Dan-Inject of North America, Fort Collins, CO, USA) into the neck musculature. Male rhinoceros SB No. 049 also received Guaifenesin (5% iv drip, Guaifenesin Injection, the Butler Company, Columbus OH, USA) during the procedure. At the conclusion of the procedure, the effects of etorphine were antagonized by IV administration of 80–200 mg naltrexone (Wildlife Pharmaceuticals). No reversal agent was used to antagonize the effects of detomidine. Blood samples were collected via venipuncture, immediately after a surgical plane of anesthesia was achieved, but before electroejaculation, and serum was harvested after centrifugation ($1200 \times g$ for 10 min) and stored ($-80\ ^{\circ}C$) until assayed.

2.3. Electroejaculation

Semen was collected from all anesthetized male Indian rhinoceroses via electroejaculation [21]. Feces were manually removed from the rectum and a rectal probe inserted, with the electrodes directed ventrally. An electrostimulator (PT Electronics, Boring, OR USA) attached to the probe was used to deliver 70–150 electrical stimuli (voltage range, 4–10 V), divided into one to five separate series. Five minute rest periods

followed each series. During each rest period, manual rectal massage was administered. Throughout the procedure, the tip of the penis was held in a warm collection cup (140 mL; VWR International, West Chester, PA, USA) to collect the ejaculate, and penile massage was performed both during stimulation and throughout the rest periods. Once the male started producing fluid, the collection cups were frequently changed. A single electroejaculation procedure was conducted on all males, with the exception of SB No. 239 ($n = 2$; 280 d interval) and SB No. 087 ($n = 4$; 280, 108, and 253 d intervals).

2.4. Ultrasonography

Either prior to or immediately following the electroejaculation procedure, transrectal ultrasonography was performed to assess the normalcy of reproductive glands and testes. A well-lubricated linear-array, transrectal ultrasound transducer (5-MHz Aloka Co. Ltd., Wallingford, CT USA) was inserted into the rectum to obtain ultrasonographic images of the prostate, bulbourethral gland, seminal vesicles, and bladder. Each testis was palpated for size and consistency, and the ultraonography was used to assess the echotexture.

2.5. Semen collection, evaluation, and processing

Semen was obtained in fractions into sequentially numbered, sterile warmed collection cups. Samples from each cup were examined microscopically (1000 and 400 X) for sperm motility (0–100%) and progressive motility status (scale of 0–5, with 0 being non-motile and 5 being rapid forward progression) [22]. For each fraction, pH was determined by pipetting 5 μL of raw semen onto an indicator strip (EM Science, Gibbstown, NJ, USA). In addition, a 100 μL aliquot was taken for subsequent osmolality testing via a vapour pressure osmometer (Wescor, Logan, UT, USA). For each ejaculate, an aliquot of raw semen (5 μL) was fixed in 0.3% (v/v) glutaraldehyde (50 μL; Sigma Aldrich, St. Louis, MO, USA) and later evaluated (100 sperm/sample) by phase-contrast microscopy (X 400–1000) for percentage of normal and pleiomorphic sperm forms [22]. In addition, aliquots of all sperm samples were fixed 1:1 with 8% (v/v) glutaraldehyde in PBS for later sperm viability assessments. Sperm fractions exhibiting similar concentrations, percent motility, and progressive motility status were combined. Sperm concentration was determined in each collection cup using a white blood cell/hemacytometer method [22]. Fractions with sperm concentrations $\geq 125 \times 10^6$/mL and ≥50% motile sperm were cryopreserved.

2.6. Sperm cryopreservation

A 2 x 2 factorial experiment was designed to determine which combination of two extenders (EQ [23] and SMEY [20]) and two cryoprotectants (glycerol and DMSO) were most effective in preserving Indian rhinoceros sperm. Semen was cryopreserved following methodology previously described [3], with a few modifications. Briefly, semen was diluted 1:1 with EQ or SMEY (two aliquots for each extender). Tubes (50 mL; VWR International) of diluted semen were transferred to a room temperature waterbath and placed in a cold room or ice filled chest (4 °C). Ice was added to the water bath over 1.5 h to gradually cool the water to 4–5 °C. After 1.5 h, samples (5 °C) were diluted 1:1 with concurrently cooled extender, containing 10% (v/v) cryoprotectant. Dilutions were made in a stepwise fashion (25, 25, and 50%, v/v) every 20 min to achieve a final concentration of 5% (v/v) cryoprotectant. Samples were then allowed to equilibrate for 45–60 min, after which sperm progressive motility was evaluated. Labeled straws were kept cool by placing them between plastic bags filled with 4 °C ice water. Semen straws (0.5 mL; AgTech, Inc., Manhattan, KS, USA) were loaded using vacuum pressure applied through a 1-mL pipette and sealed (Critoseal; Oxford Labware, St. Louis, MO, USA). A metal cane (13 mm) with attached goblet was pre-cooled to 4 °C. Straws ($n = 8$) were rapidly transferred into pre-cooled goblets on canes and immediately cyropreserved using a direct dry shipper method [24]. Briefly, a canister containing no more than eight goblets (eight straws/goblet) was rapidly lowered into a fully charged but empty dry shipper for 10 min, after which canes were plunged into liquid nitrogen (LN2).

2.7. Sperm thawing and processing

Semen was thawed by holding straws for 10 s in air (21 °C), and then immersing them in a 37 °C waterbath and shaking vigorously for 20 s. Thawed semen was emptied into a sterile pre-warmed 1.5 mL microcentrifuge tube. Thawed semen samples were examined for initial sperm motility and forward progression, and an aliquot (5 μL) of each sample was fixed for later evaluation (100 sperm/sample) of post-thaw sperm morphology. An aliquot (300 μL) of semen was placed in a microcentrifuge tube and 300 μL of warmed Sperm-Tyrode's Albumin Lactate Pyruvate (Sperm-

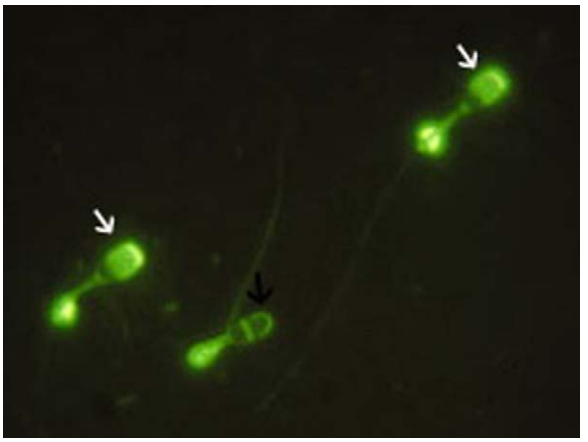

Fig. 1. Frozen-thawed Indian rhinoceros sperm stained with FITC-PNA to reveal intact (white arrow) and non-intact (black arrow) acrosomes.

TALP; [25]) medium was slowly added and then centrifuged at 600 × *g* for 3 min, the supernatant was removed, and each sperm pellet was re-suspended in 300 μL of Sperm TALP media. Re-suspended samples were examined for percent motility and forward progression. In addition, 5 μL were smeared onto a slide for acrosome assessment, 10 μL were fixed in 10 μL 8% (v/v) gluteraldehyde for viability assessment, and 5 μL were diluted in 50 μL 0.3% (v/v) gluteraldehyde for morphology assessment. Remaining aliquots of the original thawed samples were examined over time for motility. In addition, re-suspended samples were used for sperm motility, acrosome and viability assessments at 1, 3, and 6 h post thaw.

2.8. Sperm acrosome staining

Acrosomal membranes were evaluated using fluorescein-conjugated Arachis hypogea (peanut) agglutinin (FITC-PNA; Sigma; [3,24]). Sperm classified as non-intact were those with no staining, or just a single band of green at the equatorial segment, whereas sperm classified as acrosome intact exhibited staining of the entire acrosome with bright fluorescence visible only at the apical ridge (Fig. 1).

2.9. Sperm viability staining

For viability assessment, fixed samples were diluted 1:1 with Hoechst 33258 in citrate buffer and then co-incubated (5 min) while protected from light [3,24]. A wet mount was prepared on a glass slide, and at least 100 sperm/sample were evaluated (X 400–1000) using fluorescent microscopy. Sperm exhibiting bright blue staining over the head region were classified as non-viable, whereas sperm that excluded the stain in the head region were classified as viable.

2.10. Serum extraction and testosterone enzyme immunoassay (EIA)

Serum testosterone concentrations were analyzed by enzyme immunoassay [26]. Standard concentrations ranged from 3.125–200 pg/well. For this assay, polyclonal antibody R156/7 (supplied by C. Munro, University of California, Davis, CA, USA) was used at a 1:10,000 dilution with testosterone-horseradish peroxidase (HRP) added to each well at a 1:60,000 concentration. The testosterone EIA was validated for Indian rhinoceros serum by demonstrating parallelism between the standard curve and serial dilutions of pooled serum. Testosterone concentrations in Indian rhinoceros serum were determined following extraction. For this procedure, 2 mL of diethyl ether (Sigma) was added to 220 μL of serum in a 12 x 75 borosilicate glass tube (Fisher Scientific, Hampton, NH, USA) and vortexed for 45 s. The serum layer was frozen (-80 °C; 15 min) and the ether layer was immediately transferred to a new clean glass tube and dried under a stream of air. Dried samples were reconstituted in 220 μL EIA buffer and vortexed briefly. Extracted serum samples were assayed at a 1:8 dilution in buffer and serum testosterone concentrations (ng/mL) were calculated based on an extraction efficiency of 85%. The CV's for the testosterone assay were 9.5 and 12.7% for internal controls at 70 and 28% binding, respectively.

2.11. Statistical analyses

The Sigma Plot/Sigma Stat software program (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Pearson product moment correlation was used to compare serial dilutions of pooled Indian rhinoceros serum with the standard curve for the testosterone EIA. Spearman correlation was performed to determine if a relationship existed between testosterone concentrations and the age of the male, whether the male was proven or not, and total number of sperm/ejaculate collected. All percentage data (motility, acrosome status, viability, and morphology) were arcsine transformed before analysis. Standard descriptive statistics were used to summarize results and all data were expressed as mean ± SEM. Possible differences in sperm parameters (motility, progressive

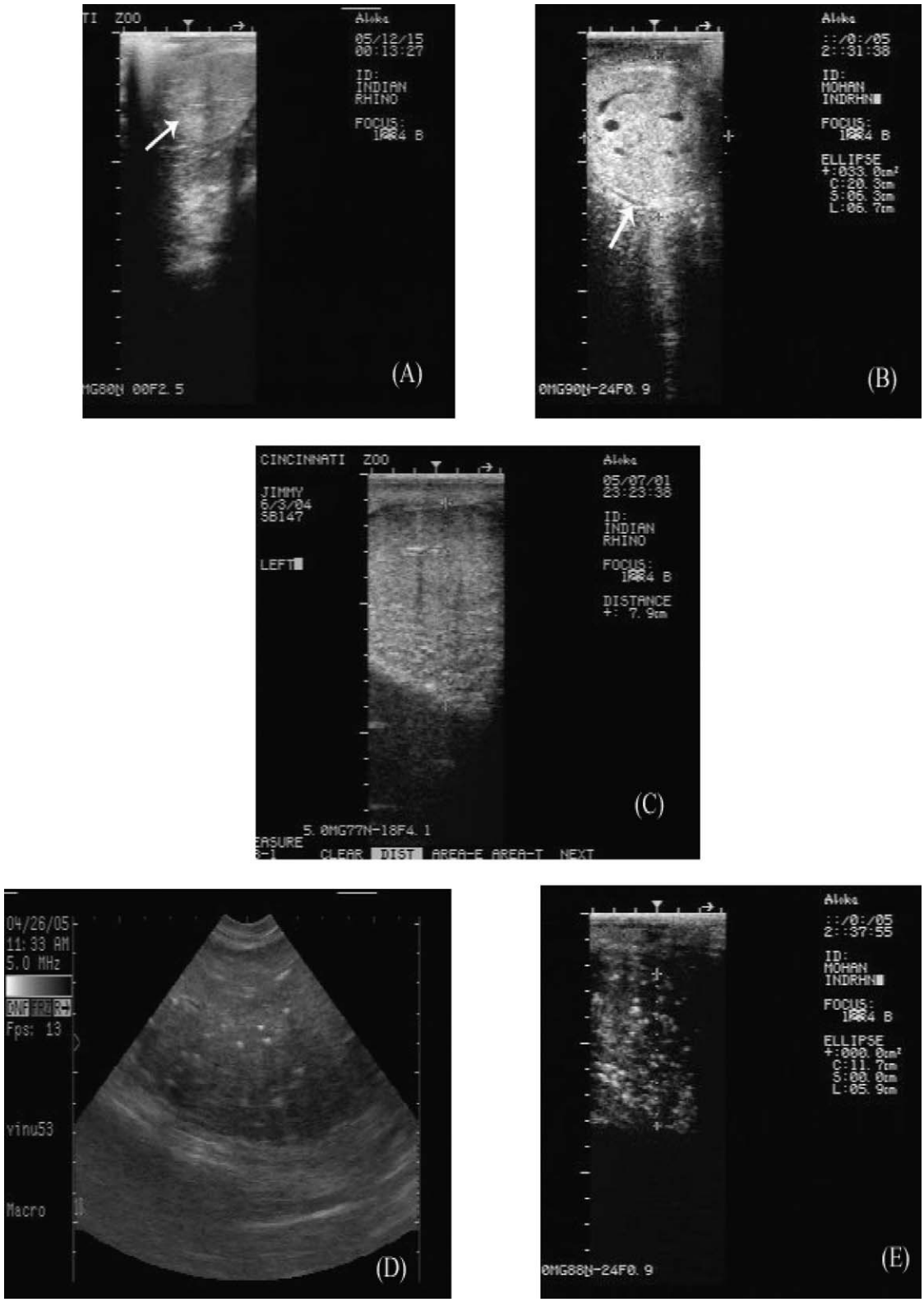

Fig. 2. Bulbourethral glands (white arrow) in male Indian rhinoceroses prior to (A) and following (B) electroejaculation. Note the increased echodensity and fluid accumulation resulting from stimulation of the gland (B). Although there were no apparent differences among male Indian

motility status, acrosome status, viability, and morphology) among the different cryoextender and cryoprotectant treatment groups over time were analyzed by repeated measures ANOVA. Pair wise multiple comparison procedures were carried out using Tukey's test for each time point to determine which treatment group(s) had significant effect(s) on a specific sperm end point. Significance was established at $P < 0.05$.

3. Results

3.1. Ultrasonography

Echodensity of the accessory sex glands were uniform and nonpathologic in appearance. Ultrasonographic imaging of Indian rhinoceros testicles revealed observational differences in the echogenicity and uniformity of the parenchyma dependent with age (Fig. 2; [27]). Specifically, hyperechodense spots within the testicular parenchyma increased in number and distribution in older animals (Fig. 2).

3.2. Semen collection

Electroejaculation procedures conducted on six male Indian rhinoceroses resulted in a total of 10 ejaculates (Table 1). However, a single male Indian rhinoceros (SB No. 087) electroejaculated on four separate occasions produced semen fractions that met the criteria for cryopreservation during only two of the four procedures. Therefore, the cryopreservation study comprised semen fractions collected from six males and eight separate ejaculates (Table 2).

Sperm concentration was high, with most males producing ejaculates with $>170 \times 10^8$ sperm/mL. Ejaculates exhibited high numbers of viable sperm with moderate progressive motility, but the majority of sperm were structurally abnormal (primarily secondary abnormalities; Table 1). The most common abnormalities included sperm with bent midpieces (with and without cytoplasmic droplets) and bent tails. There were no significant correlations in the number of motile sperm/ejaculate and the age of the male ($P = 0.462$) or whether the male was of proven versus unproven status at collection ($P = 0.381$).

Because semen samples were obtained in fractions during electroejaculation, seminal parameters varied among fractions collected for a single ejaculate. Only fractions in each ejaculate with the highest progressive sperm motility, concentration, and morphology were cryopreserved (Table 2).

3.3. Sperm cryopreservation

Although male Indian rhinoceroses produced, on average, >150 mL of semen during an electroejaculation procedure, <20% of that total volume was high quality semen appropriate for cryopreservation. Good quality fractions typically did not contain the highest sperm concentrations. Compared to fresh ejaculate fractions, motility of sperm was reduced ($P < 0.05$) following cryodilution and slow cooling prior to freezing (Fig. 3A). Whereas the progressive status of sperm in SMEY containing cryoprotectant was lower ($P < 0.05$) than that in the fresh ejaculate prior to freezing, sperm in EQ containing cryoprotectant did not differ from the fresh ejaculate (Fig. 3B).

Characteristics of frozen-thawed sperm were affected by extender type ($P < 0.05$). Specifically, higher percentages ($P < 0.05$) of progressively motile (Fig. 3A and B), viable (Fig. 4A), and acrosome-intact (Fig. 4B) Indian rhinoceros sperm were observed immediately post-thaw (0 h) when semen was cryopreserved in EQ versus SMEY extender. Post-thaw motility of sperm in EQ remained significantly higher than SMEY at all time points examined (Fig. 3A). However, the progressive status of sperm differed ($P < 0.05$) between extender treatment groups up to 3 h post-thaw. At 6 h post-thaw, the progressive status of sperm in EQ with either cryoprotectant was comparable to SMEY with DMSO, and only sperm in EQ with glycerol had higher progressive status ($P < 0.05$) than those in SMEY with glycerol (Fig. 3B). In general, motility was reduced 44–51% and 66–75% by 6 h post-thaw from that of pre-freeze values for samples cryopreserved in EQ and SMEY, respectively. Compared with fresh ejaculates, the percentage of viable sperm after cryopreservation in EQ was ~35–39% lower immediately post-thaw and following removal of extender and cryoprotectant. In contrast, percentages of viable Indian rhinoceros sperm declined ~67–70% from those recorded for fresh ejaculates when cryopreserved in SMEY and processed in the same manner post-thaw (Fig. 4A; Table 2). After 3 h post-thaw, percentages of viable sperm in EQ with either

rhinoceroses in ultrasonographic characteristics of the accessory sex glands, age-dependent changes in testicular parenchyma were observed. Note the uniform echodensity of the testicular parenchyma of a 16 y-old male Indian rhinoceros (C; SB No. 147) versus the medium (D; SB No. 053) and high degrees (E; SB No. 049) of hyperechoic spots in the testicular parenchyma of 34 y-old males.

Table 1
Reproductive characteristics of male Indian rhinoceros ($n = 6$) whole ejaculates ($n = 10$) obtained by electroejaculation.

End point	Mean ± SEM	Range
Semen volume (mL)	158.6 ± 32.86	13–337.5
Sperm motility (%)	48.0 ± 9.4	0–83
Sperm progressive motility status (0-5)	2.7 + 0.36	0–3.5
pH	8.74 ± 0.05	8.5–9.0
Normal sperm morphology (%)	38.8 ± 7.79	19–83
Primary defects (%)	0.83 ± 0.18	0.3–1.0
Secondary defects (%)	60.38 ± 7.9	17–81
Sperm concentration (X 10^8/mL)	10.78 ± 4.22[a]	1.54–34.96[a]
	10.11 ± 3.54[b]	0.005–34.96[b]
Total sperm per ejaculate (X 10^8)	304.28 ± 40.9[a]	532–171.22[a]
	273.9 ± 47.56[b]	46.5–532[b]
Motile sperm per ejaculate (X 10^8)	207.51 ± 45.45[a]	478.8–98.61[a]
	166.01 ± 45.29[b]	0–478.8[b]
Viability (%)	89.5 ± 1.99	84–95
Acrosome intact (%)	85.5 ± 1.59	74–98
Osmolality (mmol/kg)	308.04 ± 4.793	272–410
Testosterone (ng/mL)	2.08 ± 0.355	0.9–4.0
Age at collection (y)	17.5 ± 2.97	7.5–34
No. of stimuli	120.3 ± 8.6	70–150
No. of series	3.9 ± 0.277	2–5
No. of semen fractions	10.9 ± 1.21	6–8
Time of procedure (min)	36.9 ± 3.57	14–60

[a] Values represent the exclusion of $n = 2$ ejaculates that did not meet criteria for cryopreservation.
[b] Values represent the inclusion of $n = 2$ ejaculates that did not meet criteria for cryopreservation.

cryoprotectant were comparable to SMEY with DMSO and were higher ($P < 0.05$) than SMEY with glycerol (Fig. 4A). During all time points examined, there were no differences in percentages of progressively motile or viable sperm within extender treatment groups (EQ or SMEY) associated with the use of glycerol or DMSO as the cryoprotectant. However, the integrity of acrosomal membranes appeared more stable over time with the cryoprotectant DMSO, but only when used in combination with EQ extender. Although sperm extended in SMEY initially exhibited lower post-thaw acrosomal integrity compared with EQ, these effects were mitigated over time.

Among extender and cryoprotectant treatment groups, post-thaw values for percentage of morphologically normal sperm did not differ ($P \geq 0.05$). In addition, there were no differences ($P \geq 0.05$) for any group in the proportion of morphologically normal sperm in fresh versus frozen-thawed preparations.

3.4. Testosterone EIA

There was a parallel dose response between serially diluted (neat to 1:64) male Indian rhinoceros serum samples and testosterone standards throughout the range of the curve. The r between the standard and pooled serum was 0.996 ($P < 0.05$). Mean serum testosterone concentration in male Indian rhinoceroses was 2.08 ng/mL (Table 1). There were no significant correlations in serum testosterone concentrations and: 1) the age of the male ($P = 0.153$); 2) proven versus unproven status of the male ($P = 0.58$); and 3) the total numbers of sperm in the ejaculate ($P = 0.098$).

4. Discussion

This was the first comprehensive semen cryopreservation study conducted in the Indian rhinoceros. Prior to this study, there were two reports of semen collection in Indian rhinoceroses by manual massage [28] or electroejaculation [21], but neither report included data on semen cryopreservation. Although there are a few reports of successful sperm cryopreservation in other rhinoceros species, the studies have largely been based on semen collected from a single male [3,7], with the exception of two large scale studies in the African white rhinoceros (*C. s. simum, C. s. cottoni*) [8,9]. Due to minimal research on rhinoceros sperm cryopreservation, the prevalence of species-specific differences and the difficulties in freezing semen from a related perissodactyla, the domestic horse, a systematic study on Indian rhinoceros semen

Table 2
Characteristics of male Indian rhinoceros ($n = 6$) fresh ejaculate fractions ($n = 8$) obtained by electroejaculation and subsequently processed for cryopreservation.

End point	Mean ± SEM	Range
Semen fraction volume (mL)	25.69 ± 4.05	4–55
Sperm motility (%)	78.80 ± 3.31	50–90
Sperm progressive motility status (0-5)	3.45 ± 0.13	2.5–4.0
pH	8.71 ± 0.08	8.5–9.0
Normal sperm morphology (%)	56.2 ± 7.56	19–88
Primary defects (%)	0.3 ± 0.14	0–1
Secondary defects (%)	43.5 ± 7.57	11–81
Sperm concentration (X 10^8/mL)	5.16 ± 1.61	1.25–20.16
Total sperm per ejaculate fraction (X 10^8)	84.02 ± 13.46	36.8–194.72
Motile sperm per ejaculate fraction (X 10^8)	66.49 ± 1.02	18.4–138.22
Sperm viability (%)	90.4 ± 2.03	80–97
Sperm with an intact acrosome (%)	89.7 ± 3.51	85–98
Osmolality (mmol/kg)	309.75 ± 9.13	273–347

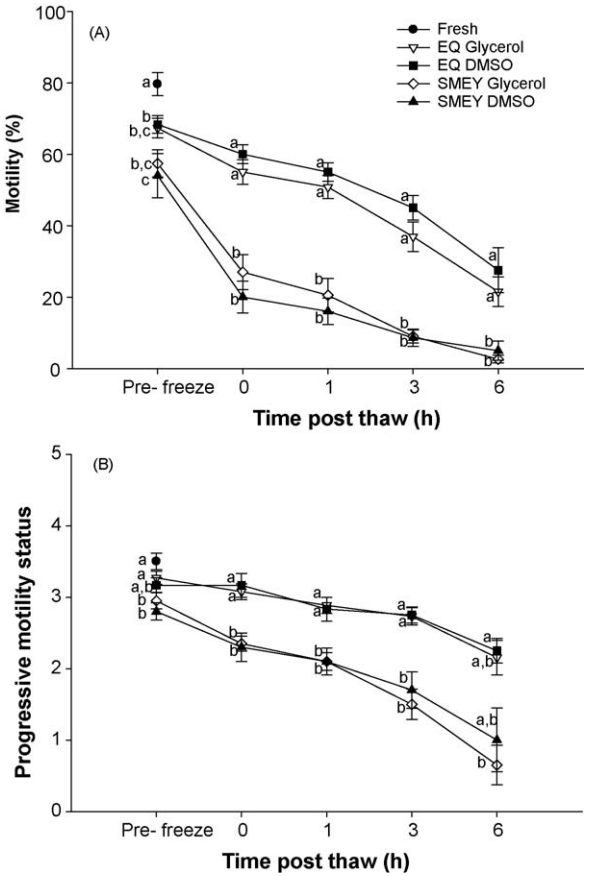

Fig. 3. Effect of extender and cryoprotectant on percentage of sperm motility (A) and progressive motility status (B) post thaw. Freshly collected sperm from Indian rhinoceroses ($n = 6$ males; 8 ejaculates) were cryopreserved in EQ and glycerol, EQ and DMSO, SMEY and glycerol, and SMEY and DMSO and later thawed to assess percentage of motile sperm and progressive motility status over time. Values are expressed as means ± SEM. [a-c]Within a time point, values without a common superscript differed ($P < 0.05$).

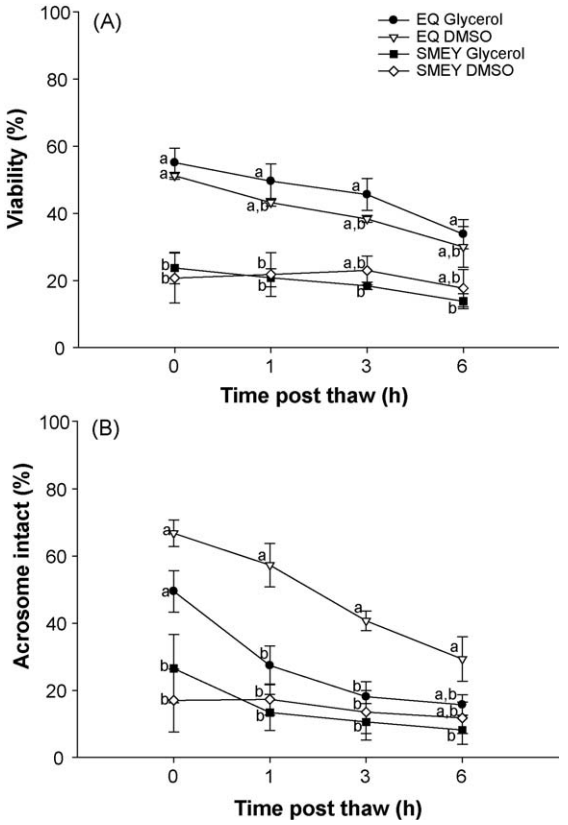

Fig. 4. Effect of cryopreservation treatment on Indian rhinoceros sperm viability (A) and acrosome status (B). Freshly collected sperm from Indian rhinoceroses ($n = 6$ males; 8 ejaculates) were cryopreserved in EQ and glycerol, EQ and DMSO, SMEY and glycerol, and SMEY and DMSO and later thawed to assess the percentage of viable sperm and sperm with intact acrosomal membranes at 0, 1, 3, and 6 h post thaw. Values are expressed as means ± SEM. [a,b]Within a time point, values without a common superscript differed ($P < 0.05$).

cryopreservation seemed essential in the establishment of an Indian rhinoceros sperm bank for use in assisted reproductive techniques.

It is broadly recognized that a single electroejaculation attempt in which a poor quality sample is obtained should never be used as grounds to label an animal infertile. This is especially true in the rhinoceros, because semen quality between males and between collection attempts in the same male can vary from high to low quality [21]. Given this history of inconsistent results, it was surprising that electroejaculation produced samples that met the cryopreservation criteria in all but two of the 10 attempts in this study. However, it was noteworthy that the two poor quality ejaculates were collected from a male that produced good quality semen fractions on two other occasions.

Although successful electroejaculation procedures have been developed in the Indian, African black, and African white rhinoceroses [8,21,28], sample quality varied substantially. It was reported that collecting in fractions improved the chance of obtaining a good quality sub-sample [21]. A similar method of collecting fractions appeared to have been adopted in a recent study of the African white rhinoceros [9], compared to an earlier non-fractionated collection technique [8]. Results of the present study reaffirmed the importance of collecting semen in fractions from rhinoceroses, due to the production of seminal fractions of variable quality. Only a small subset of seminal fractions (<20% of total volume) collected from Indian rhinoceroses by electroejaculation met the criteria for cryopreservation. We inferred that the ability to select only the good quality fractions likely contributed to our success in cryopreserving semen from this species.

Electroejaculation in Indian rhinoceroses resulted in ejaculates of high volume and sperm concentration with moderate motility. The osmolality of Indian rhinoceros electroejaculates was similar to the physiologic ejaculate of the horse [29,30] and electroejaculates of three other rhinoceros species [21]. Average pH of semen samples in our study was higher than natural ejaculates of stallions [30] and post-coital Sumatran rhinoceros semen [3]. However, our results seemed comparable to electroejaculates of other rhinoceroses suggesting that alterations in the relative composition of fluids may occur during electroejaculation versus natural ejaculation [9,21].

Indian rhinoceros electroejaculates had relatively low numbers of morphologically normal sperm, a finding consistent with previous reports for this species [21] and other rhinoceros species [3,8,9]. Similarly, stallions produce 50% morphologically normal sperm on average; percentages can vary depending on age and breeding activity [31]. Additionally, higher percentages of abnormal sperm have been associated with inbreeding [32]. The Indian rhinoceros experienced a severe bottleneck early in the 20th century to the two remaining populations in Kaziranga, Assam and Chitwan, Nepal. Moreover, most captive born Indian rhinoceroses descend from the Kaziranga population, with 48% of the genes originating from three founder individuals [12,13]. Although inbreeding may play a role in the lower percentage of normal sperm obtained from captive Indian rhinoceroses, it is likely not the primary cause. Despite the overall high percentages of abnormal sperm collected from Indian rhinoceroses, some ejaculate fractions contained significantly more normal sperm cells than other fractions within a given ejaculate, and most abnormalities were secondary in origin. Perhaps some semen fractions contained primarily aged sperm, an unnatural mix of glandular fluids that affected sperm cell structure during ejaculation, or were contaminated with low concentrations of urine that affected sperm morphology. Consistent with these theories were the high percentage of structurally normal sperm observed in epididymal samples recovered from this species [4]. Fortunately, despite low numbers of normal sperm in the initial sample, Indian rhinoceros sperm morphology was not negatively impacted further by the cryopreservation and thawing process.

There are apparently no reports regarding serum and/or fecal testosterone concentrations in captive male Asian rhinoceroses. However, fecal and serum testosterone concentrations have been reported for male African rhinoceroses [33–35]. Factors known to impact testosterone concentrations in the stallion include age, photoperiod [36], housing arrangements, and social environment [37]. Consistent with fecal testosterone results in African rhinoceroses [33], in the present study, there were no differences in serum testosterone concentrations of proven versus unproven males, or between males of advanced age versus their younger counterparts. Furthermore, there were no differences among all males in serum testosterone concentrations and semen quality obtained by electroejaculation. We concluded that testosterone may not be a particularly helpful tool in evaluating male reproductive success and physiological deficiencies may not be the primary cause of reproductive failure in male rhinoceroses.

Based on studbook data, the earliest confirmed age of successful reproduction in captivity for a male Indian rhinoceros was 6 yr [10]. In this study, electroejaculation of a 7.5 yr old male Indian rhinoceros resulted in a high quality semen sample for cryopreservation, and frozen-

thawed sperm from this sample were subsequently used to achieve a pregnancy by AI [1]. To date, the oldest Indian rhino sire to reproduce in captivity is 40 yr [10]. Age-related changes in testicular morphology have been suspected in this species [38] and confirmed in the African white rhinoceros [8]. Although we observed medium to high degrees of hyperechodense spots in the testicular parenchyma of older male Indian rhinoceroses, good quality electroejaculates were obtained.

In most mammalian cryopreservation protocols, glycerol is considered the standard cryoprotectant [39,40]. However, glycerol is toxic to sperm from some stallions [17], and successful cryopreservation is better achieved using low concentrations (2.5–6%) of this cryoprotectant [14,41–43]. Pilot studies on semen cryopreservation in rhinoceros species produced mixed results with regard to glycerol toxicity. Early on, glycerol toxicity was suspected in the African white rhinoceros [5], but further research did not substantiate that finding [6,7]. Meanwhile, neither glycerol nor DMSO appeared toxic to sperm from a Sumatran and an African black rhinoceros [3]. However, species and/or individual differences may influence sperm sensitivity to glycerol [3]. In addition, sperm from different sources (epididymal versus ejaculated) may display different sensitivities to cryoprotectant [3]. Therefore, glycerol and DMSO were chosen as the two cryoprotectants for comparison in this study.

The cryoprotectant concentration tested in this study (5% v/v) was slightly lower than the 6.25% (v/v) DMSO concentration used for African white rhinoceroses [8,9], but the same as that used to cryopreserve African black and Sumatran rhinoceros sperm [3]. Cryoprotectants are added to sperm extenders to reduce ice formation and osmotic situations during freezing, and osmotic tolerance of sperm varies among species. Low membrane permeability, coupled with the high molecular weight of glycerol, imparts high osmotic stress and limited tolerance on equine sperm, but reduced osmotic stress can be achieved with other cryoprotectants, e.g., DMSO [44]. Based on the current study, in contrast to their domestic relative, Indian rhinoceroses produced sperm that were not particularly sensitive to glycerol, as there were few or no differences in post-thaw end points following cryopreservation in glycerol and DMSO.

The FITC-PNA stain has been used previously for acrosome staining and assessment of Sumatran [3], African black [3], and African white [9] rhinoceros sperm. Therefore, FITC-PNA was employed in this study to assess the acrosome status of Indian rhinoceros sperm. Surprisingly, the staining pattern of Indian rhinoceros sperm differed from other rhinoceros species, with bright fluorescence being localized only at the apical ridge area of the sperm head. This staining pattern was also observed in Indian rhinoceros epididymal sperm obtained by gamete rescue (Fig. 5; online version only). Therefore, the staining pattern appeared to be a species-specific characteristic, and not an artifact caused by seminal plasma interference of stain uptake.

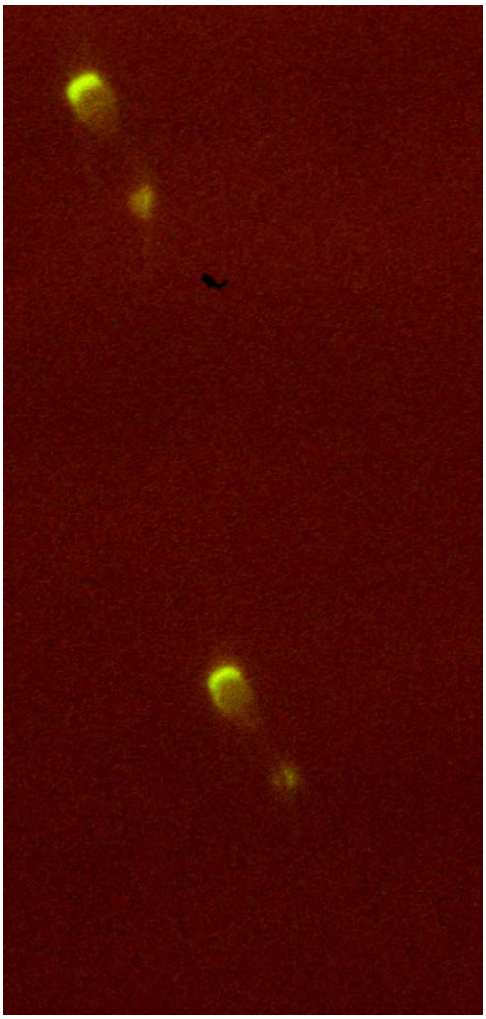

Fig. 5. (online version only). Indian rhinoceros epididymal sperm stained with FITC-PNA to reveal intact acrosomes.

Although stallion sperm are highly sensitive to cold shock [45], similar effects on sperm of African and Sumatran rhinoceros species have not been reported [3,8,9]. In the current study, Indian rhinoceros sperm diluted in SMEY were more sensitive to cooling than sperm in EQ. Our interpretation was that egg yolk was important in protecting the membrane of rhinoceros

sperm, since EQ contained a 10-fold higher percentage of egg yolk than SMEY. In fact, cryoextender type was the primary factor affecting post-thaw Indian rhinoceros sperm quality; higher post-thaw parameters exhibited by sperm cryopreserved in EQ versus SMEY could all be attributed to increased amounts of egg yolk in the extender. It is well documented that egg yolk helps maintain sperm motility after cryopreservation in many species, including the stallion [15,17,18,20,46]. Alternatively, differences in sperm quality could be attributed to differences between extenders in osmolality. Post-thaw survival of equine sperm was affected by extender osmolality [29]; in this study, the osmolality of EQ extender was higher than that of SMEY.

Although evaluating sperm characteristics pre- and post-thaw is very valuable when studying cryopreservation methods, confirming that sperm are fully functional and capable of fertilizing oocytes is a more definitive test. In domestic species, these tests are relatively easy to conduct, whereas in endangered species, similar opportunities are extremely rare. Recently, a successful AI technique for this species demonstrated that semen samples cryopreserved in EQ with glycerol or DMSO could be thawed and used to produce embryos *in vivo* [1].

In conclusion, the outcome of this study was establishment of a successful semen cryopreservation protocol for the Indian rhinoceros. Unlike its closest domestic relative, the horse, Indian rhinoceros semen appeared relatively tolerant of the cryopreservation procedure. Although semen sample quality can be compromised by high numbers of morphologically abnormal sperm, during most electroejaculation procedures, high quality semen fractions were obtained and would be appropriate for cryopreservation. Given that the numbers of frozen-thawed, motile sperm used in successful AI procedures in Indian rhinoceroses has been reported [1], and because large volumes of highly concentrated semen can be collected and cyropreseved from this species, many AI attempts could be conducted from samples obtained during a single electroejaculation procedure. In addition, the cryopreservation protocol developed in this study was both mobile and field feasible. Finally, a valuable by product resulting from this study was the first Indian rhinoceros sperm bank proven ready for use in assisted reproductive procedures.

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