

Post-coital sperm recovery and cryopreservation in the Sumatran rhinoceros (*Dicerorhinus sumatrensis*) and application to gamete rescue in the African black rhinoceros (*Diceros bicornis*)

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Sumatran rhinoceros (*Dicerorhinus sumatrensis*) sperm samples were collected from a post-copulatory female and characterized to determine their potential for sperm preservation and future use in artificial insemination. Five samples of acceptable quality from one male were used to compare the effect of two cryoprotectants (glycerol and dimethyl sulfoxide (DMSO)) and two post-thaw protocols (untreated and glass wool column) on sperm quality. The percentage of motile spermatozoa, sperm motility index (0–100) and sperm morphology were evaluated subjectively, and viability and acrosomal status were assessed using fluorescent markers. Evaluations of frozen-thawed spermatozoa were performed over a 6 h incubation interval. Post-coital semen samples ($n = 5$; 104.0 ± 9.1 ml; $2.5 \pm 0.8 \times 10^9$ total spermatozoa; mean \pm SEM) exhibited a sperm motility index of 56.7 ± 3.3 , and contained $40.2 \pm 6.3\%$, $72.0 \pm 3.2\%$ and $79.8 \pm 6.5\%$ normal, viable and acrosome-intact spermatozoa, respectively. Glycerol and DMSO were equally effective as cryoprotectants and, regardless of post-thaw protocol, samples retained greater than 80% of all pre-freeze characteristic values. Processing semen samples through glass wool yielded higher quality samples, but only half the total number of motile spermatozoa compared with untreated samples. High values for pre-freeze sperm characteristics were also maintained after cryopreservation of epididymal spermatozoa from one black rhinoceros (*Diceros bicornis*) using the same protocol. In summary, Sumatran rhinoceros spermatozoa of moderate quality can be collected from post-copulatory females. Rhinoceros sperm samples show only slight reductions in quality after cryopreservation and thawing and have potential for use in artificial insemination.

Introduction

Evolutionary mechanisms have resulted in diverse demographic and physiological features among the five species in the Rhinocerotidae family. However, with the exception of the South African white rhinoceros (*Ceratotherium simum*), all are listed as endangered with extinction by the World Conservation Union (IUCN, 1996). The Sumatran rhinoceros (*Dicerorhinus sumatrensis*) is the most endangered of all five species with a population of fewer than 400 worldwide (Foose and Reece, 1998). Together, the continued decline of Sumatran rhinoceroses in the wild and the lack of reproductive success among captive animals depict a bleak outlook for the future of this species. Captive breeding efforts have been hindered by a lack of knowledge about the reproductive physiology of this species, aggressive behaviour between pairs introduced for breeding and an insufficient number of animals. Artificial insemination (AI)

with cryopreserved spermatozoa may help to overcome the last two challenges.

The utility of AI in the propagation and genetic management of several endangered species has been demonstrated, but its success relies upon an understanding of female reproductive physiology and the ability to collect and cryopreserve semen (Monfort *et al.*, 1993; Wildt *et al.*, 1995; Wildt and Roth, 1997). AI has not been reported in any rhinoceros, but with recent advances in understanding female reproductive function in several rhino species (Sumatran: Roth *et al.*, 1998a; black: Schwarzenberger *et al.*, 1993; white: Radcliffe *et al.*, 1997; Schwarzenberger *et al.*, 1998), it may be a significant step closer to becoming a reality for this taxon. However, information on sperm collection and cryopreservation is scarce (Young, 1967; Platz *et al.*, 1979; Schaffer and Beehler, 1988), and this is likely due, in part, to the difficulties in applying common semen collection methods to this taxon.

Traditional semen collection methods that do not require anaesthesia (manual massage, artificial vagina) have been used successfully in horses (Asbury and Hughes, 1964), the closest domestic relative of the rhinoceros, and also in a

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Revised manuscript received 23 August 1999.

pachyderm, the elephant (Schmitt and Hildebrandt, 1998). However, successful collection of rhino semen by penile massage can require intensive animal conditioning, and success is highly dependent on the animal's temperament; very few animals reliably produce good quality samples (Schaffer and Beehler, 1988; Schaffer *et al.*, 1990). Collection by artificial vagina is largely unsuccessful (Young, 1967; Schaffer and Beehler, 1988; Schaffer *et al.*, 1990) and electroejaculation has been performed (Platz *et al.*, 1979; Schaffer *et al.*, 1990) but its variable success combined with the need for anaesthesia in most cases limit its use. Kuederling *et al.* (1996) reported a new method of sperm collection in marmoset monkeys. Spermatozoa were collected from the vagina of post-copulatory monkeys and offspring were produced after AI with frozen-thawed post-coital samples (Morrell *et al.*, 1998). Thus, considering the difficulties associated with rhino semen collection, recovery of post-coital semen was investigated as a method for obtaining samples that could be used in developing cryopreservation protocols.

Currently, there are no standard semen cryopreservation protocols for the rhinoceros, probably because semen collection techniques have not yielded sufficient quality samples to enable methods to be tested (Platz *et al.*, 1979; Schaffer *et al.*, 1990). Variable post-thaw sperm motility (15–70%) reported in preliminary attempts to cryopreserve rhino spermatozoa (Platz *et al.*, 1979; Schaffer and Beehler, 1988) indicate that the rhinoceros may be similar to its relative the horse; equine semen is characteristically difficult to cryopreserve (Graham, 1996). Furthermore, results from a preliminary study in the white rhinoceros indicate that glycerol is toxic to spermatozoa (Williams *et al.*, 1995). Therefore, the development of a cryopreservation protocol for rhino spermatozoa should include testing different cryoprotectants and evaluating several sperm characteristics, such as viability and acrosomal status, in addition to motility.

The Sumatran rhinoceros was the targeted species in this study. There is only one male in the USA, thus it is critical that semen from this animal is collected and cryopreserved in preparation for the time when AI might be the only option available for producing offspring in this species outside of Asia.

The specific objectives of the present study were: (i) to collect and characterize post-coital sperm samples from a Sumatran rhinoceros; (ii) to test two cryoprotectants (glycerol and DMSO) for the ability to maintain pre-freeze sperm characteristics after thawing; (iii) to compare the effects of two post-thaw sample protocols (untreated and glass wool column); and (iv) to demonstrate use of the cryopreservation protocol for sperm rescue in another rhino species.

Materials and Methods

Animals

This study was conducted with the only male Sumatran rhinoceros in the USA, maintained at the Cincinnati Zoo and Botanical Garden. The animal was wild-caught as an adult

and is estimated to be > 20 years old. The male was housed adjacent to two female Sumatran rhinoceroses and introduced for natural matings with one female during her oestrus. This female was wild-caught as a juvenile and was raised in captivity. She was 7 years old and nulliparous. Copulation between the male and female resulted in fertilization and early embryo development (based on rectal ultrasonography) indicating that the male was fertile.

Epididymal spermatozoa from a captive born African black rhinoceros (age 10 years) was obtained post-mortem. The animal had been healthy and died suddenly during transport. The animal had sired one calf.

Semen and sperm evaluations

Unless stated otherwise, all chemicals and stains were obtained from Sigma Chemical Co. (St Louis, MO). Ultrapure water for preparation of media was obtained using an ultrafiltration high-purity ion-exchange system (Nanopure; Barnstead, Dubuque, IA).

Semen pH was assessed by evaluating the colour change after placing 5 µl semen on an indicator strip (EM Science, Gibbstown, NJ). Osmolality was determined using a vapour pressure osmometer (Wescor, Logan, UT). Sperm concentration was determined using a haemocytometer after immobilizing 1 µl of the sperm suspension in 9 µl of 4% (w/v) saline solution.

The percentage of motile spermatozoa was estimated subjectively to the nearest 5% on heated slides (37°C) by examining several fields using phase-contrast microscopy (Olympus, Tokyo; × 400). Forward progressive motility was also assessed (0–5 scale; 0 = no movement, 5 = rapid forward progression), and a sperm motility index was calculated (sperm motility index = (percentage of motile spermatozoa + (forward progressive motility × 20)) ÷ 2) (Howard *et al.*, 1986).

For sperm morphology analysis, 10 µl semen was placed in 10 µl of 8% (v/v) glutaraldehyde fixative. Spermatozoa (100 per sample) were examined by phase-contrast microscopy (× 1000) for gross structural abnormalities. In addition, sperm dimensions were determined on 30 cells (10 spermatozoa per collected sample, $n = 3$ and $n = 1$ samples for Sumatran and black rhinoceros, respectively) using an eyepiece graticule and a slide micrometer (× 1000). On one occasion, cytological examinations using Giemsa staining were performed to identify non-sperm cells in post-coital samples.

Sperm viability was assessed within 24 h of fixation using the fluorescent Hoechst bisbenzimidazole 33258 (H33258) exclusion stain (De Leeuw *et al.*, 1991). Unstained spermatozoa (those with intact plasma membranes that excluded the stain) were considered viable. Briefly, 3 µl fixed spermatozoa (as described for sperm morphology) was placed in 3 µl H33258 (10 µg ml⁻¹) for 10–30 s at room temperature and protected from light. Fluorescent labelling of spermatozoa was observed using a Zeiss Axiophot microscope equipped with phase-contrast and epifluorescent optics (Carl Zeiss, Oberkochen). The excitation beam was passed through a 340–380 nm band pass filter and emitted

fluorescence was detected through a 420 nm band pass filter. Spermatozoa with bright blue fluorescence over one-half or over the entire head region were considered nonviable. Unstained spermatozoa and those showing only a pale blue staining or a thin line of blue fluorescence only at the equatorial segment were considered viable. A total of 100 spermatozoa per sample were examined ($\times 400$).

Acrosomal status was evaluated using fluorescein isothiocyanate-conjugated *Arachis hypogaea* (peanut) agglutinin (FITC-PNA) as described for scimitar-horned oryx spermatozoa (Roth *et al.*, 1998b). Sample aliquots (5 μ l) were smeared across slides and air-dried. Slides were stored in the dark at 4°C and stained within 48 h. For staining, 20 μ l FITC-PNA (100 μ g ml⁻¹) was gently spread across the slide and, after a 20–30 min incubation in a dark humidified container at 4°C, slides were rinsed in 50 ml PBS (4°C) and allowed to dry protected from light. Just before assessment, a drop of mounting medium consisting of 90% (v/v) glycerol in PBS solution and 0.1% (w/v) *p*-phenylenediamine was placed on the slide and compressed firmly with a coverslip. For evaluating fluorescent labelling of spermatozoa, the excitation beam was passed through a 450–490 nm band pass filter and emitted fluorescence was detected through a 510 nm band pass filter. Spermatozoa were considered acrosome-intact when the acrosome stained bright green, whereas those with no staining or a single band of green fluorescence at the equatorial segment were classified as non-intact (acrosome reacted or damaged). A total of 100 spermatozoa per sample were examined ($\times 1000$).

Experiment 1: collection and assessment of post-coital Sumatran rhino spermatozoa

After 6 months of trying unsuccessfully to condition a male Sumatran rhinoceros for semen collection by manual penile massage and artificial vagina, the feasibility of collecting post-coital spermatozoa was investigated. Spermatozoa were collected from a female rhinoceros 20–180 min after copulation. While the animal was lying down, a long pole with a specimen collection vessel (Fisher Scientific, Pittsburgh, PA) attached to the end was extended between the bars of the indoor stall, and the collection vessel was positioned below the vulva to capture the semen as it slowly drained from the reproductive tract. Sperm evaluations were performed within 30 min of collection. When samples were of acceptable quality (sperm motility index >40, viability >70%) they were processed for cryopreservation as outlined in Expt 2.

Experiment 2: cryopreservation of post-coital Sumatran rhino spermatozoa

Experiment 2 was a 2 \times 2 factorial design comparing the effect of two cryoprotectants (glycerol and DMSO) and two post-thaw sperm protocols (untreated and glass wool column) on sample quality. Three post-coital samples were used. The cryoprotectants glycerol and DMSO were selected due to their effective use as cryoprotective agents for

mammalian spermatozoa. In addition, in a preliminary toxicity trial, no evidence of cryoprotectant-related sperm toxicity was found during a 6 h incubation at 4°C, 21°C and 39°C in cryodiluent consisting of 5% cryoprotectant.

Aliquots of the post-coital samples were placed in 15 ml tubes and washed by centrifuging twice at 1500 *g* for 10 min to remove accessory fluid and to concentrate the spermatozoa in the sample. The sperm pellets from all tubes were combined and diluted with HEPES-Tyrode's albumin lactate pyruvate (TALP) medium (Parrish *et al.*, 1986) without glucose and supplemented with 0.6% (w/v) BSA (Fraction V; Sigma A-9418), 125 iu penicillin ml⁻¹ and 38 iu streptomycin ml⁻¹ to reduce the viscosity of the final sample (2:1.2; sperm sample:HEPES-TALP). Samples were diluted 1:1 with a standard equine semen extender (EQ; Martin *et al.*, 1979) containing 5.5% (v/v) lactose, 0.25% (w/v) disodium EDTA, 20% (v/v) egg yolk, 1.5% (w/v) glucose, 0.25% (v/v) sodium triethanolamine lauryl sulphate, 25 iu penicillin G ml⁻¹ and 25 iu streptomycin ml⁻¹ (pH 7.6 \pm 0.2, osmolality 345 \pm 5 mosm kg⁻¹) and filtered through a 0.22 μ m filter (Acrodisc). Diluted semen samples were transferred to a waterbath (21–25°C) and placed in a cold room (4°C). After 2 h, cooled samples (7°C) were diluted (1:1) with concurrently cooled standard equine semen extender containing 10% cryoprotectant. Dilutions were made in a step-wise fashion (25%, 25%, 50% of volume) at 20 min intervals. The final sample consisted of one part semen to three parts standard equine semen extender with a final cryoprotectant concentration of 5%. After equilibration for 1 h, pre-freeze sperm evaluations were conducted, and samples were loaded into 0.5 ml straws (AGTECH, Manhattan, KS) and sealed (CritoSeal; Oxford Labware, St Louis, MO). Straws were frozen in a dry shipper according to Roth *et al.* (1999). Briefly, a canister containing no more than three goblets (4–5 straws per goblet) was lowered into the core of a fully charged but empty dry shipper for 10 min then plunged into liquid nitrogen. On one occasion, ten straws (five per cryoprotectant) were frozen by placing them on a block of dry ice for 10 min (in a cold room) and then plunging them into liquid nitrogen.

Semen was thawed by holding straws for 5 s in air (21°C) and then immersing them in a 50°C waterbath and shaking vigorously for 10 s. Cooling, freezing and thawing rates were determined using a thermocouple inserted into a straw containing the cryodiluent ($n = 3$ straws). For post-thaw analyses, thawed samples ($n = 2$ straws per treatment; four straws per ejaculate) were divided into untreated and glass wool protocols. For processing through glass wool, two aliquots (100 μ l) per sample were each diluted with 400 μ l HEPES-TALP medium, and passed through a glass wool column. Columns were prepared by loosely packing 0.3 ml glass wool (Sigma) into a 1 ml syringe. They were autoclaved and rinsed three times with 1 ml HEPES-TALP just before use. The sample was collected into 1.5 ml tubes and centrifuged at 600 *g* for 3 min. The supernatant was removed leaving a 50 μ l pellet containing spermatozoa. Aliquots of untreated samples or samples processed through glass wool were added to 50 μ l drops of TALP medium without glucose (Parrish *et al.*, 1988) at a final concentration of 1.5×10^6 motile spermatozoa ml⁻¹ and incubated (39°C, 5% CO₂ in air).

For each sample, sperm were evaluated for the following characteristics at the indicated times: sperm motility index and viability, before freezing, 0, 3 and 6 h after thawing; concentration and morphology, before freezing, 0 h after thawing; acrosomal status, before freezing, 0 and 6 h after thawing.

Experiment 3: cryopreservation of epididymal black rhino spermatozoa

Attempts ($n > 10$) to collect semen by penile massage on two black rhinoceroses housed at the Cincinnati Zoo and Botanical Garden were unsuccessful, but epididymal spermatozoa were collected from a different black rhinoceros 30 h post-mortem. The testes had been removed from the animal 4 h post-mortem and stored at 4°C until sperm collection. Caudal epididymides were dissected from surrounding connective tissue and blood vessels. Epididymal tubules were sliced repeatedly with a scalpel and the sperm suspension was extruded using forceps. This collection method minimized the amount of tissue and blood cells in the final sperm suspension. During the collection procedure, the thick sperm suspension was diluted with Hepes-TALP. Before cryopreservation, samples were assessed for sperm motility index and concentration, and sperm viability, morphology and acrosomal status (200 spermatozoa per sample). Spermatozoa were extended in standard equine semen extender containing glycerol or DMSO and cryopreserved by dry shipper and dry ice methods as described in Expt 2 (2×2 factorial). Pre-freeze and post-thaw sperm evaluations were conducted according to Expt 2 except that post-thaw processing through glass wool was not used.

Statistical analysis

Data were analysed by ANOVA (Minitab Statistical Package, Release 10.1, State College, PA) and means were compared using Students *t* tests or least significance difference tests. Percentage data were subjected to arcsin transformation before ANOVA. For all analyses, $P < 0.05$ was considered significant.

Results

Experiment 1: collection and assessment of post-coital Sumatran rhino spermatozoa

A total of five Sumatran rhino sperm samples were collected after seven copulations (each during a different oestrus). On two occasions, prolonged standing of the female after copulation prevented sample recovery. Overall, samples were of moderate quality; both percentage motility and viability were $> 60\%$, but large numbers of morphologically abnormal spermatozoa were present (Table 1). The most common morphological abnormalities were misshapen heads and cytoplasmic droplets. A relatively high proportion of spermatozoa were acrosome-intact.

Two of the five sperm samples were excluded from Expt 2 due to poor quality. Both samples contained large numbers of leucocytes ($> 30\%$ of total cells in the sample) and showed low sperm motility index (≤ 30) and viability ($\leq 50\%$). The total length of Sumatran rhino spermatozoa was $42.0 \pm 0.3 \mu\text{m}$ (head length: $5.2 \pm 0.07 \mu\text{m}$; head width: $3.0 \pm 0.02 \mu\text{m}$; midpiece length: $6.8 \pm 0.1 \mu\text{m}$; tail length: $30.0 \pm 0.2 \mu\text{m}$; Fig. 1).

Experiment 2: cryopreservation of post-coital Sumatran rhino spermatozoa

After combining sperm pellets from all tubes after centrifugation, sample volume for cryopreservation was $5.8 \pm 1.2 \text{ ml}$ (approximately 200×10^6 spermatozoa ml^{-1}). During the dilution, cooling and equilibration phases, sperm viability and acrosome status remained unchanged and sperm motility index decreased only slightly (raw: 56.7 ± 3.3 ; pre-freeze: 50.0 ± 2.9).

The dry shipper cryopreservation method and thawing protocol comprised four phases: (i) slow cooling rate (25°C to 7°C ; $0.2 \pm 0.01^\circ\text{C min}^{-1}$); (ii) moderate freezing rate (4°C to -169°C ; $17.2 \pm 0.2^\circ\text{C min}^{-1}$); (iii) transfer to liquid nitrogen (-180°C to -196°C); and (iv) rapid thawing (-196°C to $25.3 \pm 2.0^\circ\text{C}$; $856.1 \pm 8.5^\circ\text{C min}^{-1}$). The time required for samples to cool from 7°C to -70°C was similar for both freezing methods (dry ice: $4.5 \pm 0.8 \text{ min}$; dry shipper: $4.8 \pm 0.2 \text{ min}$), but just before transfer of straws to liquid nitrogen, dry shipper samples reached -180°C whereas dry ice samples remained at -70°C .

Immediately after thawing (0 h) and after 6 h incubation at 39°C , sperm characteristics were similar for glycerol and DMSO so data were combined (Table 2). Overall, post-thaw Sumatran rhino samples maintained $83.5 \pm 1.8\%$, $80.2 \pm 4.2\%$ and $91.9 \pm 2.8\%$ of their pre-freeze sperm motility index, viability and acrosome integrity, respectively, when cryopreserved in a dry shipper. For the ejaculate, which was frozen by both dry ice and dry shipper methods, sperm characteristics for post-thaw samples at each time assessment were similar for both cryoprotectants and freezing methods (data not shown).

Sperm motility index values and the percentage of viable and acrosome-intact spermatozoa at 0 and 6 h were higher ($P < 0.05$) for frozen-thawed samples processed by glass wool compared with untreated samples (Table 2), but the glass wool treatment yielded only half the total number of motile spermatozoa ($5.0 \pm 1.0 \times 10^6$ versus $10.3 \pm 1.3 \times 10^6$ spermatozoa ml^{-1} , respectively). The number of viable and acrosome-intact spermatozoa in untreated samples decreased ($P < 0.05$) over the 6 h incubation period but remained relatively stable for glass wool samples. Regardless of post-thaw protocol, sperm motility index values decreased ($P < 0.05$) over time. Compared with untreated samples immediately after thawing, glass wool processing had no effect on the number of morphologically normal spermatozoa (Table 2). However, 95–100% of leucocytes were removed after glass wool processing of frozen-thawed samples (number of leucocytes per 100 spermatozoa: untreated, 22.4 ± 6.4 ; after glass wool processing, 1.0 ± 0.7).

Table 1. Characteristics of post-coital Sumatran rhinoceros semen

Parameter	Mean \pm SEM ($n = 5$ samples)
Semen characteristics	
Semen volume recovered (ml)	104.0 \pm 9.1
Osmolality (mosm kg ⁻¹)	284.3 \pm 1.3
pH	7.9 \pm 0.1
Sperm concentration ($\times 10^6$ ml ⁻¹)	24.6 \pm 8.0
Total spermatozoa recovered ($\times 10^9$)	2.5 \pm 0.8
Sperm characteristics	
Motility (%)	60.0 \pm 3.2
Forward progressive motility (0–5) ^a	2.7 \pm 0.1
Sperm motility index ^b	57.0 \pm 2.0
Viability (%)	72.0 \pm 3.2
Acrosome intact (%)	79.8 \pm 6.5
Morphologically normal (%)	40.2 \pm 6.3
Morphologically abnormal (%)	
Abnormal head ^c	33.2 \pm 2.1
Bicephalic	0.8 \pm 0.4
Coiled flagellum	1.8 \pm 0.4
Biflagellate	0.4 \pm 0.2
Abnormal midpiece	1.6 \pm 0.7
Bent midpiece	6.8 \pm 3.1
Bent flagellum	0.6 \pm 0.4
Cytoplasmic droplet	14.2 \pm 5.4
Spermatid	0.4 \pm 0.4

^aForward progressive motility of spermatozoa graded subjectively: 0 = no movement, 5 = rapid steady forward progression.

^bSperm motility index = (percentage motility + (progressive motility \times 20))/2.

^cIncludes microcephalic and macrocephalic.

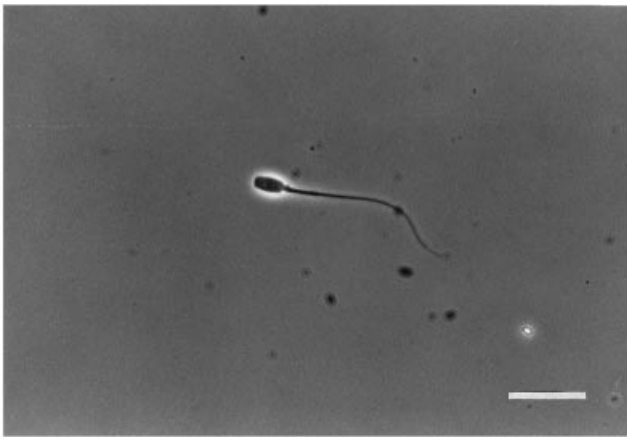


Fig. 1. Phase-contrast micrograph of a Sumatran rhinoceros spermatozoon. Scale bar represents 15 μ m.

Experiment 3: cryopreservation of epididymal black rhino spermatozoa

Spermatozoa collected from the epididymides of a black rhinoceros 30 h post-mortem were immotile at the time of collection, but motility could be induced by warming samples to 37°C (motility: 60%; status: 3.5; sperm motility index: 65). Large numbers of viable and acrosome-intact spermatozoa (93% and 95%, respectively) were observed at collection. A total of 10% of spermatozoa were morphologically normal and 82% contained a cytoplasmic droplet, but were otherwise normal. A total of 60% of

spermatozoa had an abaxially placed midpiece, a characteristic commonly observed in stallions (Long *et al.*, 1990). The total length of spermatozoa was $55.1 \pm 0.1 \mu$ m (head length: $6.2 \pm 0.06 \mu$ m; head width: $3.0 \pm 0.02 \mu$ m; midpiece length: $8.0 \pm 0.03 \mu$ m; tail length: $40.9 \pm 0.1 \mu$ m) and all dimensions with the exception of head width were larger ($P < 0.05$) than those of spermatozoa from the Sumatran rhinoceros. Sperm staining patterns with FITC-PNA were similar for black and Sumatran rhinoceroses; an example is shown (Fig. 2).

The final 5 ml concentrated sperm sample isolated from the epididymides consisted of 2 ml sperm exudate and 3 ml HEPES-TALP (concentration: 400×10^6 spermatozoa ml⁻¹). Similar to Sumatran rhino spermatozoa, only minimal loss of sperm motility index was observed during the dilution, cooling and equilibration phases with either cryoprotectant (raw: 65; pre-freeze: 60). Sperm characteristics for post-thaw samples were similar between freezing methods so data were pooled to compare cryoprotectants (Table 3). The sperm motility index was lower ($P < 0.05$) for samples cryopreserved with glycerol compared with DMSO (Table 3), but decreased similarly over time; sample sperm motility index was reduced by 12.5 and 11.8, respectively, at 6 h. Across treatments, post-thaw spermatozoa maintained $86.5 \pm 3.7\%$, $98.4 \pm 1.1\%$ and $99.7 \pm 0.3\%$ of pre-freeze sperm motility index, viability and acrosome integrity, respectively (data combined for all treatments). Although sperm motility index, percentage viability and acrosome-intact spermatozoa decreased ($P < 0.05$) during the post-thaw incubation interval, moderate values for all characteristics were still observed at 6 h (Table 3).

Table 2. Effect of processing through a glass wool column on Sumatran rhinoceros sperm quality after post-coital collection and cryopreservation ($n = 3$ ejaculates)

Sperm characteristic (number of samples)		Sample protocol	
		Untreated	Glass wool
Pre-freeze* ($n = 6$)			
Sperm motility index		50.0 \pm 2.9	n/a
Percentage viable		81.5 \pm 2.4	n/a
Percentage acrosome intact		87.0 \pm 2.4	n/a
Post-thaw† ($n = 12$)			
Percentage morphologically normal	0 h	28.6 \pm 0.8	29.9 \pm 2.5
Sperm motility index	0 h	41.6 \pm 1.2 ^a	60.9 \pm 2.8 ^b
	3 h	38.1 \pm 1.2 ^a	50.0 \pm 2.0 ^b
	6 h	29.2 \pm 1.2 ^a	42.3 \pm 2.1 ^b
	6 h	29.2 \pm 1.2 ^a	42.3 \pm 2.1 ^b
Percentage viable	0 h	64.3 \pm 2.4 ^a	74.8 \pm 3.5 ^b
	3 h	53.7 \pm 3.8 ^a	68.2 \pm 3.3 ^b
	6 h	48.1 \pm 2.9 ^a	70.9 \pm 3.0 ^b
	6 h	48.1 \pm 2.9 ^a	70.9 \pm 3.0 ^b
Percentage acrosome intact	0 h	76.8 \pm 2.5 ^a	86.7 \pm 1.4 ^b
	3 h	76.8 \pm 2.5 ^a	86.7 \pm 1.4 ^b
	6 h	69.7 \pm 2.2 ^a	83.2 \pm 2.5 ^b

Values are means \pm SEM.

*Data from diluted, cooled and equilibrated samples (data combined for cryoprotectant).

†Dry shipper freezing method only (data combined for cryoprotectant).

^a^bValues with different superscripts within the same row are significantly different ($P < 0.05$).

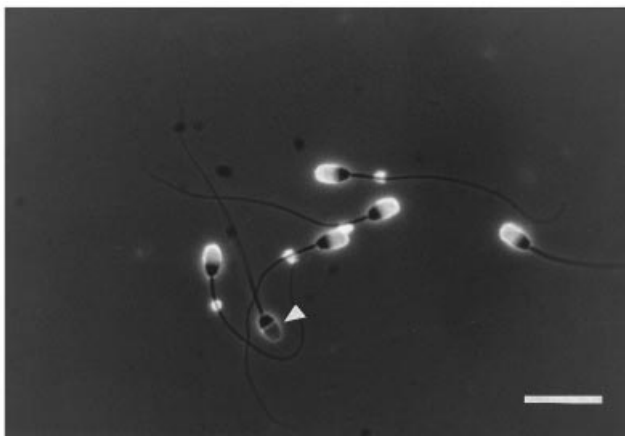


Fig. 2. Phase-contrast micrograph of black rhinoceros spermatozoa after staining with fluorescein isothiocyanate-conjugated *Arachis hypogaea* (peanut) agglutinin (FITC-PNA). Five spermatozoa with intact acrosomes show bright fluorescence over the anterior head region, and a spermatozoon without an acrosome shows slight fluorescence only along the equatorial segment of the head (arrowhead). Scale bar represents 15 μ m.

Discussion

Unique challenges of reproductive research in non-domestic animals often necessitate the development of alternative methods for conducting standard procedures. The present study describes a new method for obtaining rhino spermatozoa by non-invasive collection from a post-copulatory female. This procedure allowed the collection of multiple samples containing adequate numbers of motile, viable and acrosome-intact spermatozoa. As a result, for the first time, material was available for systematic testing of sperm cryopreservation protocols in the rhinoceros. The

results indicate that even post-coital sperm samples of moderate quality can be cryopreserved successfully.

Traditional methods of semen collection often require anaesthesia and are of limited success (Platz *et al.*, 1979; Schaffer *et al.*, 1990). Therefore, semen collection from post-coital females may be the only effective and acceptable sperm recovery method for some male rhinoceroses. It should be possible to apply this post-coital collection method to all rhinoceros species, providing post-copulatory females can be managed appropriately. In addition to providing a source of spermatozoa for genome resource banking and future AI attempts, post-coital collection provides the opportunity to confirm that a male of unknown fertility is producing motile spermatozoa. The fertility of male rhinoceroses has been questioned because there are pairs of African white and black rhinoceroses breeding repeatedly without producing calves (Roth and Brown, 1999).

One advantage to collecting this type of sample is that it represents a sample of a natural ejaculate, whereas the small volumes of fluid emitted during manual stimulation or electroejaculation may not consist of the appropriate mixture of seminal fluids. The large volume (> 100 ml) of the samples collected in the present study compared with those reported for other methods (0.7–47 ml; Schaffer *et al.*, 1990) support this suggestion. Since specific components of seminal fluid can affect sperm longevity and, ultimately, fertility (Mann and Lutwak-Mann, 1981; Baas *et al.*, 1983), post-coital sperm recovery may provide samples better suited for assisted reproduction.

Despite the advantages of this alternative semen collection method, associated with it are novel features and challenges that require consideration. For example, the quality of post-coital samples may be lower than that of the initial ejaculate since many of the high quality sperm cells probably remain in the female reproductive tract to undergo transport to the

Table 3. Black rhinoceros epididymal sperm characteristics after cryopreservation in dimethyl sulfoxide (DMSO) and glycerol

Sperm characteristic (number of samples)	Cryoprotectant		
	Glycerol	DMSO	
Pre-freeze* (<i>n</i> = 1)			
Sperm motility index	60.0	60.0	
Percentage viable	90.0	92.0	
Percentage acrosome intact	96.0	94.0	
Post-thaw† (<i>n</i> = 4)			
Sperm motility index	0 h	48.1 ± 1.2 ^a	55.6 ± 1.6 ^b
	3 h	39.4 ± 1.6 ^a	45.6 ± 0.6 ^b
	6 h	35.6 ± 2.1 ^a	43.8 ± 2.4 ^b
Percentage viable	0 h	91.8 ± 0.5	89.0 ± 1.3
	3 h	73.3 ± 3.9	76.3 ± 3.0
	6 h	72.8 ± 2.8	76.0 ± 3.8
Percentage acrosome intact	0 h	97.5 ± 0.6	94.3 ± 1.5
	6 h	75.5 ± 2.2	76.0 ± 6.4

*Data from diluted, cooled and equilibrated samples.

†Data combined for dry shipper and dry ice freezing methods.

^a^bValues with different superscripts within the same row are significantly different (*P* < 0.05).

oviducts. However, inseminated equine spermatozoa take up to 4 h to complete their transport to the oviducts (Troedsson *et al.*, 1998), thus the quality of samples collected within 1 h of copulation may be representative of the quality of the initial ejaculate. In this study, the male appeared to produce ejaculates containing high proportions of spermatozoa with abnormal heads. Although it is possible that the abundance of structurally abnormal spermatozoa in post-coital samples is slightly influenced by the selective properties of the female reproductive tract (Saacke *et al.*, 1998), the high ratio of primary to secondary sperm abnormalities indicates that sample quality was poor at ejaculation.

Another challenge associated with using post-coital semen collection was an abundance of leucocytes in the samples, and sperm quality appeared inversely related to the amount of time that passed between copulation and collection. One sample collected 3 h after copulation contained very large numbers of leucocytes, and sperm viability and motility were low after centrifugation. Aitken *et al.* (1994) reported that the quality of human spermatozoa is unaffected by high concentrations of leucocytes in the ejaculate. However, in the report of Aitken *et al.* (1994), the leucocytes were of male origin. The generation of inflammatory leucocytes after copulation or AI is a natural physiological response which has been documented in several species including horses (Troedsson *et al.*, 1998). The origin of leucocytes in post-coital rhino sperm samples is unknown but is assumed to be of female origin since the numbers were higher in samples collected 2 h after copulation. Therefore, it is recommended that samples are collected as soon as possible after copulation to minimize the concentration of leucocytes and optimize sperm quality in the sample.

Although sperm samples collected from females within 1 h of copulation contained relatively small numbers of

leucocytes, the removal of these cells may be desirable if samples are to be used for IVF or for inseminating a different female. Processing of post-thaw samples through glass wool was an effective method for removing almost all leucocytes. Furthermore, the quality of the resulting sample was higher and remained higher during the 6 h incubation interval than that of untreated samples. It is unclear whether the beneficial effect of processing through glass wool was mediated through the removal of either cryodiluent components or leucocytes, or whether the glass wool column selected for higher quality sperm cells. However, glass wool samples contained only half the total number of motile spermatozoa compared with untreated samples. Therefore, glass wool processing may not be ideal when the sample is to be used in AI and a greater number of spermatozoa is required. In contrast, this procedure may be very useful for the development of an IVF system in rhinoceroses. The potential benefits of glass wool processing before cryopreservation were not investigated but certainly warrant consideration in future studies.

In contrast to results reported for epididymal spermatozoa from an African white rhinoceros (Williams *et al.*, 1995), glycerol was not toxic to Sumatran rhino spermatozoa and had no deleterious effect on motility or acrosomal status. It is possible that spermatozoa from different types of sample (epididymal versus ejaculated) show different cryoprotectant sensitivities. However, the results from the toxicity trial conducted in the present study in conjunction with post-mortem sperm rescue in an African black rhinoceros indicated that glycerol is not toxic to epididymal spermatozoa in that species. Therefore, the potential toxicity of glycerol may be species- or even individual-specific.

A sperm cryopreservation protocol consisting of a slow cooling rate, moderate freezing rate and rapid thawing maintained high values for initial (pre-freeze) sample sperm characteristics. Both glycerol and DMSO were effective

cryoprotectants for ejaculated Sumatran rhino spermatozoa. The finding that dry shipper and dry ice methods were equally effective was not unexpected considering that the cooling rates are similar to -70°C . In addition, the ability of rhino spermatozoa to tolerate variations in cooling rates below -70°C would be expected. Similar findings have been reported for scimitar-horned oryx spermatozoa (Roth *et al.*, 1999). These authors noted that the dry shipper method may be more practical under field conditions, and the same logic may apply to rhino sperm cryopreservation.

One goal for reproductive physiologists working with endangered animals is to have protocols in place for rescuing gametes from valuable individuals when they die. It was encouraging that the cryopreservation protocol developed for post-coital Sumatran rhino spermatozoa was equally effective when applied to epididymal African black rhino spermatozoa. The success of this protocol with two types of sperm samples from two different rhino species indicates that it has potential as a universal method for this taxon. Equally important was the finding that a high quality sperm sample could be harvested from the epididymis up to 30 h post-mortem. A slight difference noted between the two animals in the present study was that post-thaw sperm motility index of black rhino spermatozoa cryopreserved with glycerol was lower than that for samples cryopreserved with DMSO. Therefore, although glycerol toxicity was not observed with either animal in this study, further studies with more animals are necessary for identifying the best cryoprotectant for spermatozoa of each rhino species.

As knowledge of reproduction in rhinoceroses increases, assisted reproduction could become a reality in facilitating captive breeding programmes for this taxon. Two of the greatest obstacles in developing AI for the rhinoceros have been the inability to develop safe, reliable methods of semen collection and a lack of knowledge about rhino semen cryopreservation. This paper describes a successful non-invasive method of sperm collection in the Sumatran rhinoceros that potentially could be applied to other rhino species. In addition, this is the first systematic sperm cryopreservation study in a rhino species, and the results appear more successful than those in other case reports. Cryopreserved sperm samples showing adequate post-thaw quality are now available for use in AI trials in both Sumatran and African black rhinoceroses.

This research was funded by the International Rhino Foundation. J. K. O'Brien was supported by The Carl and Edyth Lindner Post-Doctoral Fellowship through The Cincinnati Zoo and Botanical Garden's Center for Research of Endangered Wildlife. The authors thank M. A. McRae, J. S. Romo and P. Reinhart at the Cincinnati Zoo and Botanical Garden for assistance with Sumatran rhino sperm collection.

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