Sperm collection in rhinoceros via urethral catheterization

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ABSTRACT

Assisted reproductive technologies are playing an ever-increasing role in the management of *ex situ* rhinoceros populations. Traditionally, sperm collection in rhinos is conducted via electroejaculation (EEJ) or gamete rescue. Sperm collection via urethral catheterization (UC) is gaining popularity in wildlife reproductive sciences as it requires less specialized equipment, is faster, and less stimulatory than EEJ. This study investigated the use of UC to collect sperm from Southern white (*Ceratotherium simum simum;* n = 3) and Eastern black (*Diceros bicornis michaeli;* n = 6) rhinoceros as an alternative or complementary to collection via EEJ. UC samples (n = 14) exhibited high sperm concentration ($1.6 \pm 0.49 \times 10^9$ sperm/mL) and low volume (0.98 ± 0.26 mL). Samples obtained via EEJ (n = 11) were of moderate sperm concentration ($0.14 \pm 0.05 \times 10^9$ sperm/mL) and high volume (30.6 ± 7.2 mL). UC samples were processed for cryopreservation in OptiXcell extender. Motility of UC sperm decreased post-thaw ($56 \pm 2.4\%$) over pre-freeze ($77.1 \pm 3.2\%$; $P < 0.05$), with longevity data consistent to prior results published for sperm collected via EEJ. These results suggest sperm quality and cryo-survival are not impacted by the collection method. Since EEJ was conducted after UC, sperm parameters could not be compared between the two methods. Results indicate that UC is a viable alternative to EEJ for sperm collection in rhinoceros.

1. Introduction

Over the last century, worldwide numbers of rhinoceros plummeted from an estimated 500,000 individuals in the early 1900 s to less than 28,000 in 2023, due to poaching and habitat loss/fragmentation [1–3]. Significant concerns among conservationists exist regarding long-term population viability and management of the now fragmented populations, both *in situ* and *ex situ*. Assisted reproductive technologies (ART) can facilitate sustainable management of endangered and threatened species and are playing an ever-increasing role in the management of rhinoceros *ex situ* [4–10]. Advancements in techniques including hormone monitoring, ultrasonography, semen cryopreservation, and artificial insemination (AI) have not only improved our understanding of rhinoceros reproductive biology, but have also made it possible for rhinoceros in managed care to reproduce posthumously [6] and contribute to improved breeding management aimed at creating a more genetically sound population.

AI benefits management by minimizing health and welfare risks associated with introducing mates and providing a more economical alternative to animal transport for breeding purposes. In rhinoceros species under managed care, AI has become more successful, particularly in the last 15 years [4–6,8,9], as the field gained better understanding of species-specific estrous cycle dynamics [11–13], methods to control ovarian cyclicity and ovulation [9, 14–18], and ways to diagnose and address sub-fertility [9,19,20]. However, successful AI relies on access to viable, genetically compatible sperm samples. Therefore, collection and cryopreservation of sperm from more individuals is a priority for maintaining genetic diversity and sustainability of *ex situ* rhinoceros populations.

Sperm collection in rhinoceros has historically been done via gamete rescue [21] or electroejaculation (EEJ) [22,23]. The former is a last effort to extract epididymal sperm post-mortem and the latter requires significant logistical effort to coordinate the necessary equipment to manage the specialized nature of the collection. EEJ is performed on rhinoceros while under general anesthesia which carries inherent risk. Samples obtained via EEJ are generally high volume (2.8 – 338 mL) [22, 24–26], with significant volumetric contribution made by the accessory sex glands. Recovered total sperm cell numbers are variable (0.8 – 43

$\times 10^9$) but almost always sufficient for evaluation of reproductive potential, optimizing cryopreservation protocols [22,24,26–28], use in innovative research such as cytometric sperm sorting [29,30,31] and achieving pregnancies via artificial insemination [4,-6,8,9]. While this methodology of sperm collection has been utilized in field settings of numerous wildlife species including rhinos [32], examination of alternative collection procedures that reduce the time needed to obtain sufficient samples and require less specialized equipment and expertise, is warranted for the rhinoceros.

Sperm collection via urethral catheterization (UC) offers an alternative to traditional EEJ and requires less specialized equipment, is faster, and less stimulatory than EEJ. Use of UC in the rhinoceros is thought to be successful, in part, due to the use of medetomidine in the anesthetic combination used during general anesthesia [33–35]. Medetomidine is an alpha-two receptor agonist, a class of drugs that stimulates epididymal contractions thereby moving sperm into the urethra [36]. Alpha-two receptor agonists have been used as part of chemical ejaculation in horses for decades [37], but the relatively consistent use of medetomidine in anesthetic combinations for zoo and wildlife species is more recent [38,39]. The volume of the samples obtained during UC is smaller compared to EEJ samples, and the concentration is higher, mainly due to the lack of accessory sex gland secretions in UC samples [40]. Samples obtained via UC can be used for assessment of reproductive potential, sperm characterization, and cryopreservation as demonstrated by the growing number of taxa in which it is being used, including domestic cats (*Felis catus*), Asiatic black bears (*Ursus thibetanus*), giant anteaters (*Myrmecophaga tridactyla*), African lions (*Panthera leo*), black-footed ferrets (*Mustelo nigripes*), and polar bears (*Ursus maritimus*), among others [41–48]. The utility of UC for sperm collection in rhinoceros was first demonstrated in a single Eastern black rhinoceros (*Diceros bicornis michaeli*) undergoing frequent anesthetic procedures [49]. These preliminary results documented lower volume and higher sperm concentrations in samples obtained via UC compared to EEJ procedures, with sperm from both collection methods exhibiting similar post-thaw sperm parameters [49]. Findings from this case study provided impetus to explore UC sperm collection in additional individuals and rhinoceros species.

The present study aimed to 1) characterize sperm collected via UC in Eastern black and Southern white (*Ceratotherium simum simum*) rhinoceros; 2) explore the utility of rectal ultrasonography to confirm UC placement in the area of the prostatic urethra of rhinoceros; and 3) assess post-thaw sperm parameters to determine feasibility of UC sperm to be utilized in ART procedures.

2. Materials and methods

All chemicals were purchased from Sigma-Aldrich (Sigma, St Louis, MO, USA) unless otherwise specified. Media was prepared with tissue culture grade water. All procedures were reviewed and approved by IACUC protocol #210003MS at Omaha's Henry Doorly Zoo and Aquarium, as well as by each of the participating institutions.

2.1. Animals and sampling

Sperm samples (n = 14 UC, Table 1; n = 11 EEJ, Table 2) were obtained from nine adult (> 6 years of age) male rhinoceros: Eastern black rhinoceros (EBR; n = 6) and southern white rhinoceros (SWR; n = 3). The rhinoceros were housed at 8 different institutions within the United States and samples were collected throughout the year. Rhinos #1 and #3 were collected via both UC and EEJ twice during the study. UC and EEJ were conducted consecutively during anesthetic events, with UC preceding EEJ. All attempted collections via UC and EEJ were successful; however, for Rhino #1, three UC procedures took place that did not involve a subsequent collection via EEJ. Rhinoceros were anesthetized using protocols determined by the attending veterinarian at each institution and according to the need for the specific procedure. Therefore, the anesthetic protocol was not standardized across collections, but was documented. Except for Rhinoceros #1, anesthesia protocols included an ultra-potent opioid, the alpha-2 agonist medetomidine, and a sedative. Rhinoceros #1 was anesthetized with an etorphine-ketamine-azaperone combination [47].

2.2. Urethral catheterization (UC) and electroejaculation (EEJ)

Following anesthetic induction and intubation, and after the rhinoceros was considered stable, an exam of the bulbourethral, prostate, and seminal vesicles was performed via transrectal palpation and ultrasound exam with a linear (5–9 mHz, EVO, E.I. Medical, Loveland, CO, USA) or convex (3–7 mHz, EVOII, E.I. Medical) transducer [22,24]. The exterior of the penis was examined and cleaned with sterile saline and

Table 1

Sperm characteristics of samples obtained from black rhinoceros (*Diceros bicornis*; n=11 samples from 6 males; 1–5 collections/male) and white rhinoceros (*Ceratotherium simum*; n=3 samples; 1 sample/male) via urethral catheterization.

Rhino ID	Rhino Species	Volume (mL)	Total Motility (%)	Progressive Status (0−5)	Plasma Membrane Intact (%)	Morphologically Normal (%)	Sperm/ mL (x10^9)	Total Sperm (x10^9)
1[a]	*Diceros bicornis michaeli*	0.44 ± 0.13	64 ± 8.12	2.6 ± 0.25	80 ± 9.04	53 ± 6.51	1.84 ± 1.33	0.79 ± 0.44
2	*Diceros bicornis michaeli*	0.79	60	4.0	70	35	0.056	0.04
3[b]	*Diceros bicornis michaeli*	0.65 ± 0.50	75 ± 5	3.0	85 ± 7	51 ± 13	2.57 ± 2.07	2.71 ± 2.63
4	*Diceros bicornis michaeli*	0.25	80	3.5	84	70	0.624	0.16
5	*Diceros bicornis michaeli*	1.15	90	4.0	87	75	0.904	1.04
6	*Diceros bicornis michaeli*	1	85	4.0	92	78	0.865	0.87
7	*Ceratotherium simum simum*	0.8	80	3.5	89	70	1.83	1.46
8	*Ceratotherium simum simum*	3.0	80	3.5	76	80	1.35	4.04
9	*Ceratotherium simum simum*	3.3	80	3.0	86	81	2.73	9.0
ALL		0.98 ± 0.26	77.1 ± 3.2	3.46 ± 0.12	82.2 ± 2.1	61 ± 4.58	1.6 ± 0.49	1.94 ± 0.74

[a] mean SEM of n=5 procedures

[b] mean ± SEM of n=2 procedures

Table 2

Sperm characteristics of ejaculates from black rhinoceros (*Diceros bicornis*; n=8 samples from 6 males; 1–2 collections/male) and white rhinoceros (*Ceratotherium simum*; n=3 samples; 1 sample/male) collected via electroejaculation following urethral catheterization sperm collection.

Rhino ID	Rhino Species	Volume (mL)	Total Motility (%, range)	Progressive Status (0–5, range)	Plasma Membrane Intact (%)	Morphologically Normal (%)	Sperm/mL (x10⁹)	Total Sperm (x10⁹)
1[a]	*Diceros bicornis michaeli*	14.63 ± 14.38	50 – 80	3.0 – 3.5	70 ± 2.0	53 ± 21	0.057 ± 0.009	0.94 ± 0.93
2	*Diceros bicornis michaeli*	55.0	50 – 85	3.0 – 3.5	68	42	0.224	12.32
3[a]	*Diceros bicornis michaeli*	44.3 ± 27.25	40–90	2.0 – 4.0	85.5 ± 1.5	53 ± 7	0.07 ± 0.045	1.9 ± 0.1
4	*Diceros bicornis michaeli*	10.0	80	3.5	82	62	0.05	0.5
5	*Diceros bicornis michaeli*	68.0	80 – 90	4.0	84	69	0.077	5.25
6	*Diceros bicornis michaeli*	28.0	80 – 90	3.5 – 4.0	—	70	0.046	1.29
7	*Ceratotherium simum simum*	28.3	80	3.0 – 3.5	82	70	0.136	3.86
8	*Ceratotherium simum simum*	18.5	70 – 80	3.0 – 3.5	70	78	0.197	3.65
9	*Ceratotherium simum simum*	11.0	90	4.5	91	83	0.594	6.53
ALL		30.6 ± 7.2	-	-	79.6 ± 2.5	62.8 ± 4.6	0.14 ± 0.05	3.56 ± 1.06

[a] mean ± SEM of n=2 procedures

gauze. A sterile 5 or 7 Fr bullet tip (Adept Vet LLC, Milwaukee, WI, USA) or open tip (Equivet Kruuse 240611/Henry Schein, Melville, NY, USA) catheter, with a small amount of non-spermicidal gel applied to the tip, was passed through the opening of the urethra to the prostate (~ 89–110 cm). Placement was determined by the feeling of resistance against the movement of the catheter due to constriction near the prostate and confirmed by transrectal ultrasound (Fig. 1). Once proximal to the prostatic urethra, a 1–3 mL syringe was used to apply slight negative pressure to the catheter by pulling the plunger back to ~0.5 mL for ~1 min. Negative pressure was relieved, and the catheter was gently removed from the urethra. Prior to catheter removal, except in Rhinoceros #1, a sharpie pen was used to mark the catheter at the location of the urethral opening and the distance of collection for each catheter was measured to the nearest cm. Sample was expelled from the lumen of the catheter into a microcentrifuge tube by pushing air through the lumen with a syringe; samples were stored in an insulated container until processing.

EEJ procedures were conducted as previously described for each species [26,31]. Rectal probes and an electroejaculator were used to deliver 50–90 stimuli of 2–8 V (35–355 mAmps) over the course of 2–3 series (~20–30 stimuli/series; Supplementary Table 1). Each series was followed by a 5 min rest period during which the quality of any obtained

Fig. 1. Ultrasonographic image of urethral catheterization in a male Eastern black (*Diceros bicornis michaeli*) rhinoceros. The tip of the catheter (red arrows) was inserted until it approached the area of the prostate gland (white arrows). Scale bars denote 1 cm increments.

sample was assessed stall-side. Samples were collected into sterile Whirl Pak(R)) bags (Nasco, Fort Atkinson, WI, USA) and stored in an insulated container until assessment.

2.3. Sperm evaluation

Total percent motility and progressive status (0–5; 5 = motile sperm displaying rapid forward progression) were assessed for each sample obtained. Samples collected via the same method that displayed similar attributes of moderate to high quality (>60% motile and progressive status of 3) were combined. Overall concentration of the obtained sample was calculated using a hemocytometer. Viability (plasma membrane integrity), morphology, and pH of the samples were assessed. To determine viability (proportion of sperm with an intact plasma membrane), 5 μL of sample was stained with ~5 μL of eosin-nigrosin live/dead exclusion stain (Jorgensen Laboratories, Inc., Loveland, CA, USA), smeared across the surface of a slide, allowed to dry and assessed under 400x brightfield magnification (200 spermatozoa/sample). Full exclusion of the dye indicated viable sperm. An aliquot of sample (10uL) was fixed in 0.4% glutaraldehyde for use in morphological assessment. The fixed sample was assessed under 400x magnification with phase contrast and 200 spermatozoa per slide were evaluated as previously described [26,31]. pH was determined using pH strips (MColorHast™ Merck, Darmstadt, Germany/EMD Millipore Corp. Billerica, MA).

2.4. Sperm cryopreservation and thawing

Sperm collected via EEJ was cryopreserved as previously described [24,26]. Briefly, sample was diluted in either egg yolk-based extender (EQ; Rhinoceros #1) or commercially available OptiXcell (IMV Technologies U.S.A, Maple Grove, MN, USA; Rhinoceros #2–9). EQ was generated using lactose (5.5% v/v), disodium EDTA (0.25% w/v), egg yolk (20% v/v), glucose (1.5% w/v), Equex STM (0.25%v/v; Nova Chemical, Moon Township, PA 15108 USA), 25 iu penicillin G mL^{-1}, and 25 iu streptomycin mL^{-1} [21,50], Samples extended with EQ were cooled to 4°C for 1.5 h, diluted 1:1 with EQ + 10% glycerol stepwise (25, 25, 50% every 20 min), and equilibrated for 1 h at 4°C. OptiXcell was prepared as per manufacturer's instructions and used to dilute EEJ samples 1:1 prior to loading into 0.5 mL straws [26]. Given the high sperm concentrations of samples collected via UC, these samples were first diluted (minimum 1:1 v:v) in PBS (Dulbecco's PBS, Thermo Fisher
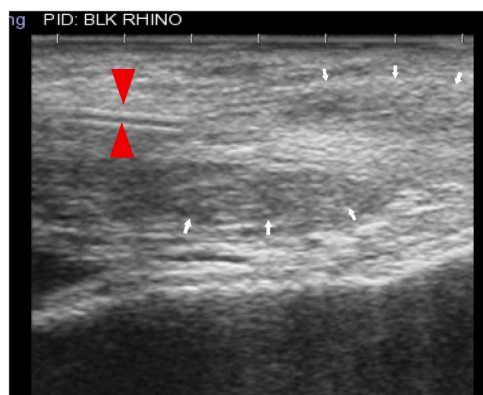
Scientific, Waltham, MA 02451 USA) or egg yolk free EQ-based media (611 mM α-lactose, 302.6 mM glucose (anhydrous), 12.65 mM sodium citrate dihydrate, 10 mM EDTA-disodium salt, 14.28 mM sodium bicarbonate, 1.2% penicillin/streptomycin (pH 7.2–7.4, 310–320 mOsm/kg) [31] before diluting 1:1 in prepared OptiXcell. Diluted samples were loaded into 0.5 mL straws and chilled to 4°C for 1 h. All straws were frozen in a charged dry shipper for a minimum of 10 min before storage in liquid nitrogen (LN_2) [21].

Samples were thawed by removing the straw from LN_2 and holding at RT (22°C) for 20 s, placing the straw in a 38°C water bath for 30 s, and transferring contents to a microcentrifuge tube. Sperm motility and morphology were evaluated post-thaw as described above for pre-freeze. A 10ul aliquot of UC and EEJ samples were submitted for anaerobic and aerobic culture (Antech Diagnostics, Fountain Valley, CA 92708). Two straws per collection method per male were evaluated at 0, 3, 6, and 24 h post-thaw. The data from replicate straws were averaged prior to analysis.

The reproductive tract of a 22 y male Eastern black was opportunistically dissected during a post-mortem following euthanasia due to causes unrelated to this study. Distance from urethral opening to widening of the lumen was measured and photo-documented.

2.5. Statistics

Descriptive statistics for viability, motility, progressive status, morphology, volume, and concentration were calculated for each collection method (UC and EEJ) pre-freeze and post-thaw. A viability value was not recorded for the EEJ sample collected from rhinoceros #6. UC and EEJ were conducted on the same day disallowing statistical comparison between collection methods. Extender comparisons have previously been reported [26] for rhino sperm collected via EEJ, therefore pre-freeze and post-thaw data obtained using this collection method are presented in Supplementary Figure 1. Differences in the percentage of viable pre-freeze and time 0 post-thaw UC sperm were evaluated using a t-test. Total motility and progressive status of UC sperm over time were analyzed by repeated measures ANOVA. Microsoft Excel and SigmaStat (SPSS Inc., Chicago, IL, USA) were used for statistical analysis. Data from the males collected more than once were averaged before analyzed. Data are presented as mean ± SEM and statistical significance was defined as $P \leq 0.05$.

3. Results

Spermic samples were obtained from all individuals via both methods, UC (Supplementary Table 2) and EEJ (Supplementary Table 3). The catheters were inserted an average of 85 ± 2.23 cm for Eastern black and 107.13 ± 3.94 cm for Southern white rhinoceros. Post-mortem assessment of the reproductive tract of a male Eastern black rhinoceros revealed the widest point of the lumen of the urethra (~43 cm from the urethral opening) was almost 1 cm in diameter (Fig. 2). Data describing quality parameters of samples collected via UC and EEJ are presented in Table 1 and Table 2, respectively.

Quality of sperm obtained via each of the two methods was similar for each of the nine rhinoceros. Cryopreservation of UC sperm resulted in a 21% ($P < 0.05$; Fig. 3A) reduction in motility at time 0 post-thaw (56 ± 2.4%). Similarly, the percentage of plasma membrane intact UC sperm at 0 h post-thaw (58.2 ± 2.2%) was 25% lower ($P < 0.05$) than prior to cryopreservation. UC sperm exhibited similar total motility between 0 h and 3 h post-thaw (Fig. 3A), whereas motility declined significantly at 6 h (39.7 ± 2.5%) and again at 24 h (16.5 ± 3.8%; Fig. 3A). The progressive status of UC sperm remained stable throughout the freeze-thaw process and did not decline until 24 h ($P < 0.05$; Fig. 3B) post-thaw.

Microbiological tests performed indicated that two samples from rhinoceros #1 had growth of *Escherichia coli*: one UC sample and one aliquot collected during EEJ the following month. The remaining samples from all other individuals were negative for microbial growth.
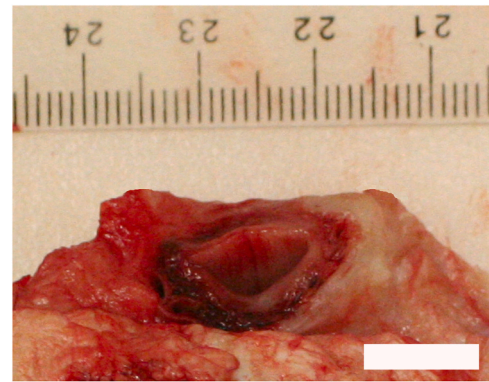

Fig. 2. Post-mortem transverse cut of the urethra of an Eastern black rhinoceros (*Diceros bicornis michaeli*) made ~43 cm from the urethral opening. Scale bar denotes 1 cm.

4. Discussion

This study demonstrated the viability of employing UC as a method for collecting sperm during routine health examinations of rhinoceros in human care. Results highlight the potential of the UC method to consistently contribute to reproductive assessments and gamete cryopreservation. Despite the comparatively lower volumetric yield of UC, the benefits of its opportunistic application, such as during routine health exams, ease of use, straight forward technology requirements, and the already routine-use of alpha-two agonists during anesthetic events, broadens accessibility for practitioners and make this an appealing method for maximizing sample diversity in genetic resource banks. UC proved to be efficient, furthering its utility in time-restricted scenarios such as *in situ*. Furthermore, the absence of accessory sex gland fluid eliminates the need for centrifugation, thereby minimizing potential insults to sperm and facilitating processing.

UC preceded EEJ for all procedures, therefore, sperm yielded by EEJ was reduced as compared to other EEJ-only reported collections. The range of total sperm counts for UC only (0.04–9.0×10^9 sperm) was comparable to previous reports of 0.5–17.4×10^9 for white rhinoceros [22,26,31] and 2.0–13.0×10^9 for black rhinoceros [26,44] collected via EEJ, demonstrating UC's potential as an alternative method to EEJ. UC samples also demonstrated similar quality parameters to samples collected via EEJ for both pre-freeze and post-thaw evaluations. In the current study, pre-freeze motility and progressive status values of UC samples ranged 60–90% and 2.6–4.0, respectively; comparable to the EEJ samples reported herein (40–90% and 2.0–4.5) and those reported in prior studies [22,26,27,31].

Combining UC with other field-friendly techniques has the potential to facilitate sperm collection *in situ* as well as making it more user-friendly for opportunistic collections at zoological institutions. For this study, samples were primarily cryopreserved using a commercially available extender, OptiXcell (IMV Technologies). As previously reported [26], OptiXcell does not contain animal products and can be transported at room temperature (RT), facilitating its use in settings where refrigeration is challenging. In addition, shorter equilibration times were shown to be just as effective as longer times in freezing rhinoceros sperm [26]. This synergy of an easier and faster sperm collection method alongside an efficient protocol for freezing and storing sperm after collection will translate well to field settings.

The lack of accessory sex gland fluid and greater sperm concentrations of the UC samples required the modification of the processing method for cryoextension in OptiXcell. Prepared OptiXcell contains 6.4% glycerol, leading to a final concentration of 3.2% glycerol once a sample is diluted 1:1, as recommended by the manufacturer. Rhinoceros semen samples are typically cryopreserved at a 5% glycerol and a concentration range of 100–400×10^6 [5,24,26,27]. In order to freeze at a
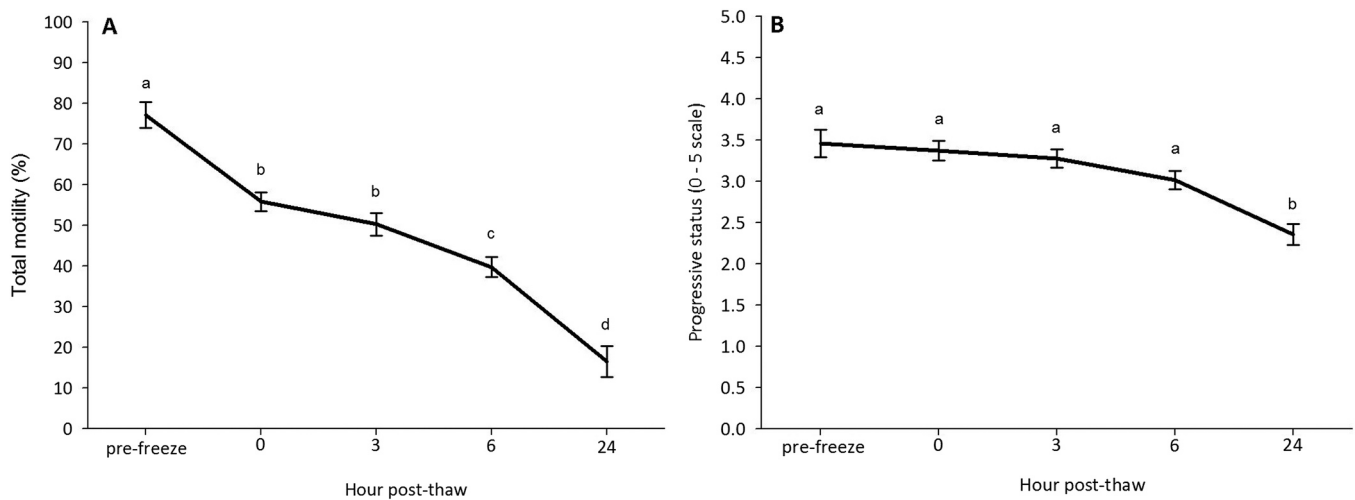
Fig. 3. Pre-freeze and post-thaw A) motility and B) progressive status of sperm collected via urethral catheterization (UC) in Eastern black (*Diceros bicornis michaeli*) and Southern white (*Ceratotherium simum simum*) rhinoceroses. Post-thaw evaluations were conducted at 0, 3, 6, and 24 h. Different letters indicate statistical significance($P < 0.05$).

similar sperm concentration, the UC samples first needed to be diluted with PBS or EQ-based media prior to equilibration in OptiXcell. Diluting the sample with OptiXcell only would result in a much higher glycerol content than is typically desired and potentially result in a detrimental impact on sperm quality and survival. The use of PBS or EQ-based diluents prior to cryopreservation did not significantly impact the cryosurvival of the samples. UC samples showed a decline in motility as a result of the freeze-thaw process that fell within the 15–30% range reported in prior rhino sperm cryopreservation studies [22, 24–27]. The lack of seminal plasma in UC samples did not negatively impact sperm quality post-thaw, consistent with freeze-thaw results of rhino samples obtained by EEJ and subjected to removal of seminal plasma prior to sperm cryopreservation [27].

UC presents a promising avenue for enhancing the accessibility of sperm collection in rhinoceros species, thereby potentially increasing the frequency of collections and promoting the addition of individuals previously unrepresented in genetic resource banks. Urethral catheterization is a technique familiar to veterinarians as it is commonly used for medical reasons. The main challenge is to insert the catheter deep enough to reach the prostate, but not so far as to reach the urinary bladder to avoid urine contamination. Resistance on the movement of the catheter due to constriction near the prostatic urethra was adequate for determining placement, which was visually confirmed via ultrasonography [22,24].

The present study did not allow for assessment of the effect of various drug combinations as these were always at the discretion of the attending veterinarian, and hence varied from case to case. In the future, studies that investigate the effect and time to effect of specific anesthetic combinations on sperm yield in a prospective manner would be of great value [31]. To date, males have required general anesthesia for semen collection, which comes with inherent risk. Utilizing UC would reduce the time necessary for sperm collection compared to EEJ. Additionally, in contrast to EEJ, the veterinary team can work on other areas while sperm samples are being collected and the UC collection process does not disrupt ECG monitoring. The shorter time necessary reduces the risk of anesthetic related problems such as myopathy. All this makes UC collection a good opportunistic procedure as it can be performed during any anesthetic event such as translocation events in the wild, pre-shipment exams, routine physical exams, or during exams to address a problem.

4.1. Limitations

As this study sampled via UC prior to EEJ within the same anesthetic event, it was not possible to compare outcomes between collection procedures. However, it was not the authors' intention to suggest one method was better than the other, but instead to demonstrate the feasibility of an alternative method for sperm collection in these species. Additionally, there was little consistency in the anesthetic combinations, preempting any conclusions about the effect of various anesthetics, also a necessary area of study.

5. Conclusion

Overall, UC offers a new alternative sperm collection procedure for rhinoceros, where spermic samples can be collected across a greater number of immobilization procedures, benefitting both the management of genetic resources and conservation efforts. In summary, small volumes of highly concentrated sperm can be recovered via UC and it is faster and less technologically complicated than EEJ.

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Monica Stoops: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Conceptualization. **Jessye Wojtusik:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Justine O'Brien:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Anneke Moresco:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use any AI-assisted technologies in the writing of this manuscript.

Declaration of Competing Interest

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.therwi.2024.100090.

References

[1] R. EmslieDiceros bicornis IUCN Red. List Threat. Species 2020. e. T6557A152728945. Accessed 05 March ,2024, , 10.2305/IUCN.UK.2020-1.RLTS. T6557A152728945.en Accessed 05 March..

[2] R. Emslie, , The IUCN Red List of threatened Species 2020. e.T4185A45813880. 2024).Ceratotherium simum.〈https://dx.doi.org/10.2305/IUCN.UK.2020-1.RLTS. T4185A45813880.en〉 (Accessed 05 March.

[3] International Rhino Foundation, State of the Rhino 2023. 〈https://rhinos. org/about-rhinos/state-of-the-rhino/〉. (Accessed 05 March 2024).

[4] T.B. Hildebrandt, R. Hermes, C. Walzer, E. Sós, V. Molnar, L. Mezösi, A. Schnorrenberg, S. Silinski, J. Streich, F. Schwarzenberger, F. Göritz, Artificial insemination in the anoestrus and the postpartum white rhinoceros using GnRH analogue to induce ovulation, Theriogenology 67 (2007) 1473–1484, https://doi. org/10.1016/j.theriogenology.2007.03.005.

[5] R. Hermes, F. Goritz, J. Saragusty, E. Sós, V. Molnar, C.E. Reid, F. Schwarzenberger, T.B. Hildebrandt, First successful artificial insemination with frozen-thawed semen in rhinoceros, Theriogenology 71 (2009) 393–399, https:// doi.org/10.1016/j.theriogenology.2008.10.008.

[6] M.A. Stoops, M.K. Campbell, C.J. DeChant, J. Hauser, J. Kottwitz, R.D. Pairan, W. Shaffstall, K. Volle, T.L. Roth, Enhancing captive Indian rhinoceros genetics via artificial insemination of cryopreserved sperm, Anim. Reprod. Sci. 172 (2016) 60–75, https://doi.org/10.1016/j.anireprosci.2016.07.003.

[7] T.B. Hildebrandt, R. Hermes, S. Colleoni, S. Diecke, S. Holtze, M.B. Renfree, J. Stejskal, K. Hayashi, M. Drukker, P. Loi, F. Göritz, Embryos and embryonic stem cells from the white rhinoceros, Nat. Commun. 9 (2018) 2589, https://doi.org/ 10.1038/s41467-018-04959-2.

[8] P.M. Pennington, K.L. Marshall, J.M. Capiro, L. Howard, B.S. Durrant, BS, Pregnancies following long luteal phases in southern white rhinoceros (*Ceratotherium simum simum*), Zoo. Biol. 39 (2019) 141–144, https://doi.org/ 10.1002/zoo.21529.

[9] M. Stoops, A. Moresco, J. O'Brien, L. Penfold, J. Gillis, J. Wojtusik, L. Metrione, Overcoming reproductive disorders in female greater one-horned rhinoceros (*Rhinoceros unicornis*) to improve artificial insemination and natural breeding success, 394-301, Clin. Theriogenology 13 (2021), https://doi.org/10.58292/ct. v13.9317.

[10] T.B. Hildebrandt, S. Holtze, S. Colleoni, R. Hermes, J. Stejskal, I. Lekolool, D. Ndeereh, P. Omondi, L. Kariuki, D. Mijele, S. Mutisya, In vitro fertilization program in white rhinoceros, Reproduction 166 (2023) 383–399, https://doi.org/ 10.1530/REP-23-0087.

[11] R.W. Radcliffe, N.M. Czekala, S.A. Osofsky, Combined serial ultrasonography and fecal progestin analysis for reproductive evaluation of the female white rhinoceros (*Ceratotherium simum simum*): preliminary results, Zoo. Biol. 16 (1997) 445–456, https://doi.org/10.1002/(SICI)1098-2361(1997)16:5<445::AID-ZOO7>3.0.CO;2- A.

[12] R.W. Radcliffe, A.I. Eyres, M.L. Patton, N.M. Czekala, R.H. Emslie, Ultrasonographic characterization of ovarian events and fetal gestational parameters in two southern black rhinoceros (*Diceros bicornis minor*) and correlation to fecal progesterone, Theriogenology 55 (2001) 1033–1049, https:// doi.org/10.1016/S0093-691X(01)00464-2.

[13] M.A. Stoops, R.D. Pairan, T.L. Roth, Follicular, endocrine, and behavioral dynamics of the Indian rhinoceros (*Rhinoceros unicornis*) oestrous cycle, Reproduction 128 (2004) 843–856, https://doi.org/10.1530/rep.1.00328.

[14] R. Hermes, T.B. Hildebrandt, C. Walzer, F. Goritz, C. Gray, C. Niemuller, F. Schwarzenberger, Estrus induction in white rhinoceros (*Ceratotherium simum*), Theriogenology 78 (2012) 1217–1223, https://doi.org/10.1016/j. theriogenology.2012.05.015.

[15] M.A. Stoops, J.K. O'Brien, J. Niederlander, L. Metrione, J. Pootoolal, K. Delk, C. Niemuller, D. Hagen, R.H. Douglas, J. Proudfoot, Administration of biorelease progesterone and estradiol or GnRH to induce estrous cycles and breeding in anestrous African white rhinoceroses (*Ceratotherium simum simum*), Proc. *6th Int. Soc. Wildl. Endocrinol. Conf.* (2017) 111.

[16] T.L. Roth, M.E. Schook, M.A. Stoops, Monitoring and controlling ovarian function in the rhinoceros, Theriogenology 109 (2018) 48–57, https://doi.org/10.1016/j. theriogenology.2017.12.007.

[17] P.M. Pennington, K.L. Marshall, J.M. Capiro, R.G. Felton, B.S. Durrant, Ovulation induction in anovulatory southern white rhinoceros *(Ceratotherium simum simum)* without altrenogest, Conserv. Physiol. 7 (2019) coz033, https://doi.org/10.1093/ conphys/coz033.

[18] R. Hermes, F. Balfanz, S. Haderthauer, E. Dungl, T.B. Hildebrandt, F. Schwarzenberger, Case Report: ovulation induction in greater one-horned rhinoceros (*Rhinoceros unicornis*), Front. Vet. Sci. 8 (2021) 657284, https://doi. org/10.3389/fvets.2021.657284.

[19] R. Hermes, T.B. Hildebrandt, C. Walzer, F. Göritz, M.L. Patton, S. Silinski, M. J. Anderson, C.E. Reid, G. Wibbelt, K. Tomasova, F. Schwarzenberger, The effect of long non-reproductive periods on the genital health in captive female white rhinoceroses (*Ceratotherium simum simum, Cs cottoni*), Theriogenology 65 (2006) 1492–1515, https://doi.org/10.1016/j.theriogenology.2005.09.002.

[20] R. Hermes, F. Göritz, J. Saragusty, M.A. Stoops, T.B. Hildebrandt, Reproductive tract tumours: the scourge of woman reproduction ails Indian rhinoceroses, PLoS ONE 9 (2014) e92595, https://doi.org/10.1371/journal.pone.0092595.

[21] J.K. O'Brien, T.L. Roth, Post-coital sperm recovery and cryopreservation in the Sumatran rhinoceros (*Dicerorhinus sumatrensis*) and application to gamete rescue in the African black rhinoceros (*Diceros bicornis*), J. Reprod. Fert. 118 (2000) 263–271, https://doi.org/10.1530/reprod/118.2.263.

[22] R. Hermes, T.B. Hildebrandt, S. Blottner, C. Walzer, S. Silinski, M.L. Patton, G. Wibbelt, F. Schwarzenberger, F. Göritz, Reproductive soundness of captive southern and northern white rhinoceroses (*Ceratotherium simum, Cs. Cottoni*): evaluation of male genital tract morphology and semen quality before and after cryopreservation, Theriogenology 63 (2005) 219–238, https://doi.org/10.1016/j. theriogenology.2004.04.007.

[23] T.L. Roth, M.A. Stoops, M.W. Atkinson, E.S. Blumer, M.K. Campbell, K.N. Cameron, S.B. Citino, A.K. Maas, Semen collection in the rhinoceros by electroejaculation with a uniquely designed probe, J. Zoo. Wildl. Med. 36 (2005) 617–627, https:// doi.org/10.1638/05-019.1.

[24] M.A. Stoops, M.W. Atkinson, E.S. Blumer, M.K. Campbell, T.L. Roth, Semen cryopreservation in the Indian rhinoceros (*Rhinoceros unicornis*), Theriogenology 73 (2010) 1104–1115, https://doi.org/10.1016/j.theriogenology.2010.01.011.

[25] J. Wojtusik, M.A. Stoops, T.L. Roth, Comparison of soy lecithin, coconut water, and coconut milk as substitutes for egg-yolk in semen cryodiluent for black rhinoceros (*Diceros bicornis*) and Indian rhinoceros (*Rhinoceros unicornis*), Theriogenology 121 (2018) 72–77, https://doi.org/10.1016/j.theriogenology.2018.07.042.

[26] J. Wojtusik, M.A. Stoops, T.L. Roth, Animal protein-free OptiXcell and shortened equilibration periods can replace egg yolk-based extender and slow cooling for rhinoceros semen cryopreservation, Cryobiology 89 (2019) 21–25, https://doi. org/10.1016/j.cryobiol.2019.06.003.

[27] R. Hermes, T.B. Hildebrandt, F. Göritz, Cryopreservation in rhinoceros – setting a new benchmark for sperm cryosurvival, PLoS One 13 (2018) e0200154, https:// doi.org/10.1371/journal.pone.0200154.

[28] J.P. Rickard, K. Pool, S.P. de Graaf, T. Portas, N. Rouke, M. Wiesner, T. B. Hildebrandt, F. Göritz, R. Hermes, Increasing the yield and cryosurvival of spermatozoa from rhinoceros ejaculates using the enzyme papain, Biology 11 (2022) 154, https://doi.org/10.3390/biology11020154.

[29] B. Behr, D. Rath, P. Mueller, T.B. Hildebrandt, F. Goeritz, B.C. Braun, T. Leahy, S. P. de Graaf, W.M.C. Maxwell, R. Hermes, Feasibility of sex-sorting sperm from the white and the black rhinoceros (*Ceratotherium simum, Diceros bicornis*), Theriogenology 72 (2009) 353–364, https://doi.org/10.1016/j. theriogenology.2009.03.001.

[30] J.K. O'Brien, K.J. Steinman, T.R. Robeck, Application of sperm sorting and associated reproductive technology for wildlife management and conservation, Theriogenology 71 (2009) 98–107, https://doi.org/10.1016/j. theriogenology.2008.09.052.

[31] J.K. O'Brien, T.L. Roth, M.A. Stoops, R.L. Ball, K.J. Steinman, G.A. Montano, C. C. Love, T.R. Robeck, Sperm sex sorting and preservation for managing the sex ratio and genetic diversity of the southern white rhinoceros (*Ceratotherium simum simum*), Anim. Reprod. Sci. 152 (2015) 137–153, https://doi.org/10.1016/j. anireprosci.2014.11.016.

[32] J. Meuffels, I. Luther-Binoir, H. Bertschinger, I. Lueders I, Semen collection for cryopreservation in live wild black (*Diceros bicornis*) and white rhinoceroses (*Ceratotherium simum*), Proc. American Association of Zoo Veterinarians / European Association of Zoo and Wildlife Veterinarians Conference (2021).

[33] S.B. Citino, Use of medetomidine in chemical restraint protocols for captive African rhinoceroses, Proc. Am. Assoc. Zoo. Vet. / Assoc. Reptil. Amphib. Vet. (2008) 108–109.

[34] R.W. Radcliffe, PvdB. Morkel, Rhinoceroses, in: G. West, D. Heard, N. Caulkett (Eds.), Zoo Animal and Wildlife Immobilization and Anesthesia 2nd Edition, John, Wiley and Sons, Inc, 2014, pp. 741–771.

[35] I.M. Mora, J.N. Langan, R.S. Bailey, C. Aitken-Palmer, M.J. Adkesson, K.N. Tang, S. K. Chinnadurai, Repeated anesthesia in a black rhinoceros (*Diceros bicornis*) to

manage upper respiratory obstruction, J. Zoo. Wildl. Med. 49 (2018) 1041–1046, https://doi.org/10.1638/2018-0095.1.

[36] A. MacDonald, J.C. McGrath, The distribution of adrenoceptors and other drug receptor between the two ends of the rat vas deferens as revealed by selective agonists and antagonists, Br. J. Pharmacol. 71 (1980) 445–458, https://doi.org/10.1111/j.1476-5381.1980.tb10957.x.

[37] A. Khanam, A.A. Swelum, F.A. Khan, Pharmacologically induced ex copula ejaculation in horses and donkeys, Front. Vet. Sci. 8, 669423. ⟨https://doi.org/10.3389/fvets.2021.669423⟩.

[38] J.P. Raath, A. Semjonov, the Selection of Anesthetic Combinations, in: E. Miller, N. Lamberski, P. Calle (Eds.), Fowler's Zoo and Wild Animal Medicine Current Therapy, Vol. 10, W.B. Saunders, 2023, pp. 355-360.

[39] L. Love, 2 Receptor agonists and antagonists, in: T. Aarnes, P. Lerche (Eds.), Pharmacology in Veterinary Anesthesia and Analgesia 1st Edition, Wiley-Blackwell, 2024, pp. 26–54.

[40] D. Zambelli, F. Prati, M. Cunto, E. Iacono, B. Merlo, Quality and in vitro fertilizing ability of cryopreserved cat spermatozoa obtained by urethral catheterization after medetomidine administration, Theriogenology 69 (2008) 485–490, https://doi.org/10.1016/j.theriogenology.2007.10.019.

[41] A. Moresco, H.L. Bateman, J. Newsom, W.F. Swanson, Effect of pellet volume and thawing temperature on vitrification efficacy with domestic cat semen collected via urethral catheterization, Reprod. Fert. Dev. 29 (2017) 137, https://doi.org/10.1071/RDv29n1Ab59.

[42] D.H. Jeong, J.J. Yang, M.K. Seo, A.N. Lee, Y.K. Lim, Effectiveness of urethral catheterization under ultrasound guidance for semen collection from Asiatic black bears (*Ursus thibetanus*), Theriogenology 129 (2019) 154–159, https://doi.org/10.1016/j.theriogenology.2019.02.032.

[43] G.R. Araujo, F.R. Miranda, T. Deco-Souza, L. Fromme, M.G. Kersul, M.C.C. Silva, A. C.B. Aguiar, P.N. Jorge-Neto, Semen collection by urethral catheterization after immobilization with medetomidine in a free-ranging giant anteater (*Myrmecophaga tridactyla*), Proc. Zoo. Wildl. Health Conf. (2019) 89.

[44] J. Meuffels, I. Lueders, H. Bertschinger, I. Luther-Binoir, F. Pohlin, L. Gerber, B. Tindall, Cardiopulmonary parameters and arterial blood gases during etorphine-medetomidine-midazolam immobilization in free-ranging black rhinoceroses (*Diceros bicornis*) undergoing electro-ejaculation - a preliminary study, Front. Vet. Sci. 8 (2021) 740614, https://doi.org/10.3389/fvets.2021.740614.

[45] I. Lueders, I. Luther, G. Scheepers, G. van der Horst, Improved semen collection method for wild felids: urethra catheterization yields high sperm quality in African lions (*Panthera leo*), Theriogenology 78 (2012) 696–701, https://doi.org/10.1016/j.theriogenology.2012.02.026.

[46] W.F. Swanson, H.L. Bateman, L.M. Vansandt, Urethral catheterization and sperm vitrification for simplified semen banking in felids, Reprod. Domest. Anim. 52 (2017) 255–260, https://doi.org/10.1111/rda.12863.

[47] P. Mackie, B. Chan, M. Franke, G.F. Mastromonaco, Urethral catheterization as an alternative method for collecting sperm in black-footed ferret (*Mustela nigripes*), Cons. Physiol. 8 (2020) coaaa078, https://doi.org/10.1093/conphys/coaa078.

[48] J. Wojtusik, T.L. Roth, E. Curry, Case studies in polar bear (*Ursus maritimus*) sperm collection and cryopreservation techniques, Animals 12 (2022) 430, https://doi.org/10.3390/ani12040430.

[49] A. Moresco, R.S. Larsen, D. Boon, J.K. O'Brien, M.A. Stoops, Semen collection in black rhinoceros (*Diceros bicornis*) via urethral catheterization, Proc. Annu. Conf. Am. Assoc. Zoo. Vet. (2015) 170–171.

[50] J.C. Martin, E. Klug, A.R. Günzel, Centrifugation of stallion semen and its storage in large volume straws, J. Reprod. Fertil. Suppl. 27 (1979) 45–51.