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Gamete rescue in the African black rhinoceros (*Diceros bicornis*)

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Abstract

Mortality rates are high among captive African black rhinoceroses (*Diceros bicornis*), due to increased susceptibility to disease. The ability to rescue genetic material from individuals that die unexpectedly represents a practical approach to assist ex situ conservation efforts. The objectives of the present study were to attempt postmortem oocyte recovery from ovaries of African black rhinoceroses (N = 6) and to test the efficacy of equine protocols for rhinoceros oocyte IVM and IVF using cryopreserved rhinoceros sperm. The interval from ovary removal to oocyte recovery was 25.3 ± 13.9 h (mean ± SD). Ovaries were transported at 4 °C or 22 °C and effects of temperature on postmortem oocyte competence was evaluated. Numbers of oocytes collected per female averaged 15.8 ± 6.9. In total, 95 oocytes were recovered. Of these, 85 were inseminated using homologous sperm and 10 were inseminated using heterologous sperm. Overall, substantial numbers of viable oocytes were retrieved from African black rhinoceros ovaries 1 to 2 days postmortem from ovaries stored at ambient temperature. A proportion of these oocytes matured and underwent penetration and fertilization by heterologous or homologous frozen-thawed rhinoceros sperm. The reproductive competence of postmortem oocytes was further demonstrated by development of a single two-cell embryo. Despite the need for further refinements, gamete rescue in the rhinoceros has promise for producing rhinoceros embryos, as well as testing sperm functions in vitro.
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Keywords: Rhinoceros; *Diceros bicornis*; Oocytes; In vitro maturation; In vitro fertilization

1. Introduction

Ex situ breeding programs have important roles in the conservation of endangered species, but only if self-sustaining and genetically healthy populations are developed. Although the captive breeding program for the critically endangered African black rhinoceros (*Diceros bicornis*) contains sufficient genetic diversity, long-term viability is threatened by high rates of mortality [1–6] and suboptimal reproduction [7].

Assisted reproductive techniques such as in IVM, IVF, embryo cryopreservation, and embryo transfer could be useful for rescuing and preserving genetic material from rhinoceroses that die unexpectedly, providing a means of infusing this unrepresented genetic material into the population. However, IVM and IVF research in the closest domestic relative of the rhinoceros, the horse, has presented challenges. To date, no foals have been produced from oocytes undergoing IVM and IVF, and only two foals have resulted from IVF embryos derived from in vivo-matured oocytes [8,9]. Only by employing intracytoplasmic sperm injection (ICSI) have fertilization and cleavage rates of equine IVM oocytes increased [10,11].

Within the past decade, tremendous achievements were made in development and application of assisted reproductive technologies for the rhinoceros. Reliable

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methods for collecting and cryopreserving sperm from both living and deceased rhinoceroses have been established [12–15]. The functionality of frozen-thawed sperm has been demonstrated through successful AI in the Indian rhinoceros [16] and subsequently repeated in the African white rhinoceros [17]. Transrectal aspiration of oocytes from African black and white rhinoceroses after hormonal superstimulation of ovarian follicles has recently been conducted [18]. These oocytes were subjected to either ICSI (using frozen-thawed sperm) or IVF (with fresh chilled sperm) [18]. That the latter resulted in a single four-cell embryo represented an important achievement in development of rhinoceros IVF [18]. However, the most prevalent source of rhinoceros oocytes will likely come from ovaries collected postmortem at unpredictable times. Therefore, a reliable and repeatable IVM and IVF procedure using postmortem oocytes and previously cryopreserved sperm will be critical in advancing reproductive technologies, while ensuring valuable genetic resources are not lost from this taxon.

The present study was conducted opportunistically to gather data on the conditions necessary to mature and fertilize oocytes of African black rhinoceroses that die abruptly, or are euthanized due to debilitating health. Our specific objectives were to: (1) develop a method of recovering viable African black rhinoceros oocytes from ovaries collected postmortem from rhinoceroses maintained at zoos across the USA; (2) assess the ability of these oocytes to mature in vitro; (3) examine the interaction of frozen-thawed rhinoceros sperm with IVM oocytes in an IVF system; and (4) monitor the development of any embryos produced in culture.

2. Materials and methods

2.1. Animals

In total, six female African black rhinoceroses (N = 4 *D.b. michaeli* and N = 2 *D.b. minor*) located at five zoological institutions were included in the study: studbook number (SB No.) 235, Chicago Zoological Society, Brookfield, IL, USA; SB No. 717 and 351, Miami Metrozoo, Miami, FL, USA; SB No. 466, Fossil Rim Wildlife Center, Glen Rose, TX, USA; SB No. 863, Milwaukee County Zoological Gardens, Milwaukee, WI, USA; SB No. 489, Potter Park Zoo, Lansing, MI, USA. Females ranged in age from 0 to 27 y and three females were of proven fertility (SB Nos. 235, 351, 466). At necropsy, ovaries were removed and individually wrapped in sterile gauze soaked in PBS, placed in a plastic bag, then sealed and labeled with left or right. Ovaries were transported to the Center for Conservation and Research of Endangered Wildlife at the Cincinnati Zoo & Botanical Garden (Cincinnati, OH, USA) and processed. The first two sets of ovaries (SB No. 235 and SB No. 717) were shipped at 4 °C. Based on the results from those first two attempts, all other ovaries (SB Nos. 466, 351, 863, 489) were shipped at room temperature (approximately 22 °C). Ovarian structures were noted and measured with the exception of SB No. 717, the stillborn calf.

2.2. Sperm cryopreservation

Epididymal sperm were recovered postmortem from three African black rhinoceroses (N = 2 *D.b. michaeli* SB No. 395, Miami Metrozoo, Miami, FL, USA; SB No. 389, the Wilds, Cumberland, OH, USA; N = 1 *D.b. minor* SB No. 403, Disney's Animal Kingdom, Orlando, FL, USA) and cryopreserved according to O'Brien and Roth 2000 [12]. In addition, sperm that had been cryopreserved from semen collected via electroejaculation of an Indian rhinoceros (SB No. 222, Bronx Zoo, NY, USA; [15]) was used for the heterologous IVF trial. Males ranged in age from 10 to 16 y; two males (SB Nos. 389, 395) were of proven fertility. Prefreeze total motility (estimated subjectively to the nearest 5% at 37 °C) of epididymal black rhinoceros sperm ranged from 60% to 70% at 2.5 to 3.0 forward progression (scale of 0 to 5, with 0 being nonmotile and 5 being rapid forward progression) [19]. The prefreeze motility of ejaculated Indian rhinoceros sperm was 75% at a 3.0 forward progression. Epididymal sperm were cryopreserved in standard equine (EQ) extender [20] at concentrations ranging from 108 to 298 $\times$ 10^6 sperm/mL [12]. The Indian rhinoceros semen was cryopreserved in EQ extender with 5% DMSO at a concentration of 192 $\times$ 10^6 sperm/mL [15].

2.3. Sperm thawing and processing

Sperm were thawed by holding straws for 10 sec in air (21 °C) and then immersing them in a 38 °C water bath and shaking vigorously for 20 sec. The content of each straw was emptied into a sterile, warmed 1.5-mL microcentrifuge tube. Samples were examined microscopically (X 100 to 400) for percent motility (0 to 100%) and forward progressive rating. To remove extender and cryoprotectant from sperm suspensions, a 1:1 dilution (v:v; 300 μL) with warm Sperm-Tyrode's Albumin Lactate Pyruvate (Sperm-TALP; [21]) medium was conducted, followed by centrifugation at 600 $\times$ g for 3 min. After centrifugation, the supernatant was removed and each sperm pellet was resuspended in

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Sperm-TALP media, and percent motility and forward progression assessed and recorded.

2.4. Oocyte recovery and IVM

This research was conducted over a 10 yr interval, with varying oocyte protocols. In general, the most recent best practices for equine IVM/IVF at the time of gamete rescue were applied. African black rhinoceros oocytes were recovered by aspirating and scraping surface follicles using a 20- to 21-gauge needle attached to a 3-cc syringe, followed by mincing ovaries with a scalpel blade in TCM-199 Washing Medium (M7653, Sigma Aldrich, St. Louis, MO, USA) containing Hank's salts (0.1 g/L L-glutamine, 0.35 g/L $NaHCO_3$, 0.1% [v:v] BSA [3311, Sigma Aldrich], 100 U/mL penicillin and 50 μg/mL streptomycin) at 37 °C. Oocytes were classified into one of three categories: grade 1, medium to darkly pigmented and completely surrounded by expanded cumulus cells; grade 2, dark to lightly pigmented surrounded by several layers of compact cumulus cells; and grade 3, dark to lightly pigmented with either no cumulus/corona radiata or only a single layer of corona radiata cells. Oocytes were washed twice in TCM-199 Washing Medium and once in TCM-199 Maturation Medium containing Earle's salts (M7528, Sigma Aldrich) supplemented with 0.1 g/L L-glutamine, 0.1% (v:v) BSA (3311, Sigma Aldrich), 100 IU/mL penicillin, 50 μg/mL streptomycin, 2 mmol/L pyruvate, and 10% (v:v) fetal calf serum.

Oocytes obtained from the first two recoveries (SB No. 235 and SB No. 717) were matured for 24 h in TCM-199 Maturation Medium with 1 μg/mL ovine FSH (NIDDK-oFSH-19), 5 μg/mL ovine LH (NIDDK-oLH-26), 1 μg/mL estradiol (E-9000, Sigma Aldrich). Oocytes obtained during the third recovery (SB No. 466) were matured for 36 h in TCM-199 with 20 μg/mL ovine FSH and 2 μg/mL estradiol. All remaining oocytes (SB Nos. 351, 863, 489) were matured for 32 h in TCM-199 Maturation Medium with 1 μg/mL estradiol (E-8875, Sigma Aldrich), 5 IU/mL porcine FSH (Sioux Biochemical, Sioux Center, IA, USA), and 10 IU/mL ovine LH. All oocytes were matured in 200 μL drops of medium (≤6 oocytes per drop), under sterile filtered washed mineral oil (M8410, Sigma Aldrich) in plastic petri dishes (39 °C, 5% CO_2 in air). Following maturation, oocytes were rinsed in 500 μL wells of HEPES-TALP [21] rinsed in equilibrated 500 μL wells of IVF-TALP [21] under oil, then transferred to 90-μL microdrops of the same medium under oil and incubated (39 °C, 5% CO_2 in air) for 1 to 4 h prior to insemination. Each fertilization drop, containing four to six matured oocytes, was inseminated with diluted sperm for a final concentration of 1.5 to 2×10^6 motile sperm/mL. Oocytes obtained during the first and third recoveries remained in the IVF drops for 24 and 6.5 h, respectively. For all other recoveries, oocytes were incubated in IVF drops for 36 to 48 h. At 36 to 48 h postinsemination (pi), oocytes were stripped of cumulus cells by mouth pipetting and assessed for cleavage. Uncleaved oocytes were transferred to fresh drops of IVF-TALP for an additional 48 h of culture, whereas any oocytes that cleaved were transferred to 500 μL wells of DMEM-F12 media (D8437; Sigma Aldrich) containing 10% (v:v) fetal calf serum. Every 48 h, half of the medium in all wells was removed and replaced with fresh medium. At 72 h pi, all oocytes were fixed in IVF-TALP containing 5% (v:v) formalin for later nuclear staining to assess maturation status.

2.5. Effect of IVM on homologous sperm binding

Because there was little sperm-oocyte interaction in the first two gamete rescue attempts, a subset of oocytes recovered from female SB No. 351 were immediately washed through HEPES-TALP and transferred to IVF-TALP drops for insemination to determine if IVM procedures were negatively impacting subsequent sperm-egg interactions. A second insemination was conducted at 32 h. The remaining oocytes underwent IVM followed by a single IVF procedure.

2.6. Heterologous IVF

To determine if heterologous IVF within the rhinoceros taxon could provide a means of testing sperm function, grade 2 oocytes recovered from SB No. 863 were inseminated following IVM using frozen-thawed Indian rhinoceros sperm. All remaining grades 1 and 2 oocytes recovered from this female underwent IVM and were inseminated using homologous sperm.

2.7. Oocyte staining

For nuclear staining, oocytes were transferred into 4-well plastic dishes (Nunc, Roskilde, Denmark) containing 500 μL of Hoechst 33342 in citrate buffer (10 μg/mL, 9.0 g/L NaCl, 4.4 g/L $C_6H_8O_7$, pH 7.0) and coincubated (20 min; room temperature (RT)) while protected from light [22]. Oocytes were transferred onto a clean glass slide and compressed using cover slips mounted with nail polish. Each slide was examined immediately using fluorescent microscopy (X 400 to 1000) to determine the number of sperm attached to or penetrating the zona pellucida of each oocyte, and to assess the nuclear maturational status (i.e., germinal

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Table 1
Results of gamete rescue in the African black rhinoceros.

Female studbook number	Age	Year	Recovery time*	Number of oocytes	Oocyte grade†	Sperm	Degenerated	GV	MI	MII	PN	Embryo	Oocytes with sperm (%)	Number of sperm bound (range)
235	27	1998	22	11	0, 8, 3	389	4	7					0	NA
717	0	1999	34	21	2, 19, 0	389	21						70	1–16
466	22	2001	12	4	1, 2, 1	389	1		2	1			NA	NA
351‡	19	2004	24	20	12, 8, 0	389	1	3	2	1		1	100	2–16
						389‡	1	10	1				100	1–18
863§	5	2006	12	21	11, 10, 0	403	8	3	8		1		82	1
						222§		1					100	1–11
489	14	2008	48	18	13, 3, 2	395	5	5	7	1			67	1–17

GV, germinal vesicle; MI, metaphase I; MII, metaphase II; NA, not applicable.
* Interval from ovary removal to oocytes in culture (h).
† Grades 1, 2, and 3.
‡ Twelve oocytes not matured.
§ Ten oocytes underwent heterologous IVF.

vesicle (GV), metaphase I (MI), metaphase II (MII), degenerated, or fertilized [23]).

2.8. Statistical analyses

The Sigma Stat software program (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Standard descriptive statistics were used to summarize results and all data were expressed as mean ± SD. To determine if numbers of sperm bound to oocytes differed depending on whether oocytes were subjected to IVM, a one-way ANOVA was performed. Significance was established at <0.05.

3. Results

3.1. Ovaries

With the exception of ovaries from the stillborn SB No. 717, ovaries of African black rhinoceroses contained small visible follicles 13–30 mm in diameter. Periovarian cysts were present in two females (SB No. 863 and 466) and measured up to 55 mm in diameter. Corpus hemorrhagicum were observed in the ovaries of three females (SB No. 351, 466, and 489).

The interval from ovarian removal to oocyte recovery varied from 12 to 48 h (Table 1). Ovaries obtained from five adult African black rhinoceroses yielded 74 oocytes (14.8 ± 7.2 per female). Percentages of oocytes classified as grades 1, 2, and 3 were 52.7 (39/74), 67.6 (50/74), and 8.1 (6/74), respectively (Table 1). The ovaries of a stillborn calf (SB No. 717) resulted in the recovery of 21 oocytes, of which only 9.5% (2/21) were classified as grade 1 (Table 1).

3.2. Oocyte maturation

Of the 95 oocytes recovered, a total of 83 oocytes were matured in culture. Evaluation of maturational status after IVF revealed 49.4% (41/83) were degenerate, 34.9% (29/83) were at the GV stage, 24.1% (20/83) were at MI, 3.6% (3/83) were at MII, 1.2% (1/83) had a single pronuclei, and 1.2% (1/83) had cleaved and contained two visible pronuclei (Fig. 1). Of the 32 oocytes recovered following chilled transport, 78.1% (25/32) were degenerate, with the remaining 21.9% (7/32) at GV stage. In contrast, ovaries shipped at RT

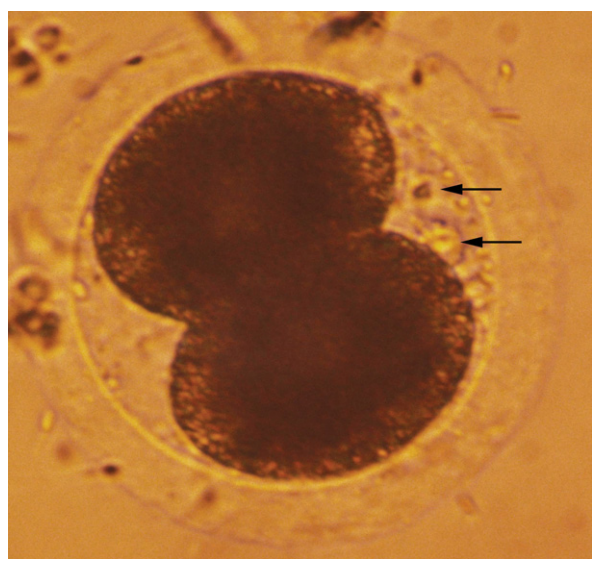

Fig. 1. Photomicrograph of a two-cell African black rhinoceros embryo with two polar bodies (arrows).

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resulted in a total of 51 recovered oocytes, of which 31.4% (16/51) were degenerate, 43.1% (22/51) GV stage, 39.2% (20/51) MI, 5.9% (3/51) MII, 2.0% (1/51) single pronuclei, and 2.0% (1/51) cleaved with two pronuclei and two polar bodies visible upon staining.

3.3. Homologous IVF

The motility of African black rhinoceros sperm declined markedly following postthaw centrifugation and resuspension in Sperm-TALP (0 h postthaw: 35%–60% progressive motility, 2.5–3.0 forward progression rating; 0 h pi: 30%–35% progressive motility, 2.0–2.5 forward progression rating). Regardless, sperm remained motile (≤ 15%) in culture up to 24 h pi.

The IVM and IVF procedures using chilled ovaries resulted in only loose binding of sperm that were lost during pipetting. However, subsequent procedures confirmed sperm could bind, penetrate, and fertilize IVM oocytes (Table 1, Fig. 2A). A single grade 2 oocyte matured and fertilized in vitro resulted in a single two-cell embryo at approximately 36 h pi (Fig 1). However, the embryo did not cleave any further in culture.

3.4. Effect of IVM on homologous sperm binding

Oocytes that immediately underwent IVF (N = 12) after recovery had 5.1 ± 1.4 sperm bound (Table 1). Oocytes subjected to IVM followed by IVF (N = 8) had 7.3 ± 1.6 sperm bound (Table 1). Numbers of sperm bound were not different (P = 0.15) between IVM oocytes and oocytes that were not matured in culture.

3.5. Heterologous IVF

Indian rhino sperm were also negatively impacted by centrifugation and resuspension in Sperm-TALP postthaw (0 h postthaw: 70% progressive motility, 3.0 forward progression rating). Motility was 30% at a 2.5 forward progression rating at 0 h pi, and declined to 10% by 24 h. The average number of Indian rhinoceros sperm bound to African black rhinoceros oocytes was 4 (Table 1). Although no oocytes cleaved, all contained sperm bound to their zonae pellucidae. Furthermore, several oocytes contained sperm within their zonae pellucidae and/or present in the perivitelline space (Fig. 2B). None of the oocytes were degenerate upon staining. In fact, only 10% (1/10) of oocytes were at the GV stage, 80% (8/10) at MI, and 10% (1/10) had a single pronuclei and was presumably parthenogenetically activated (Table 1).

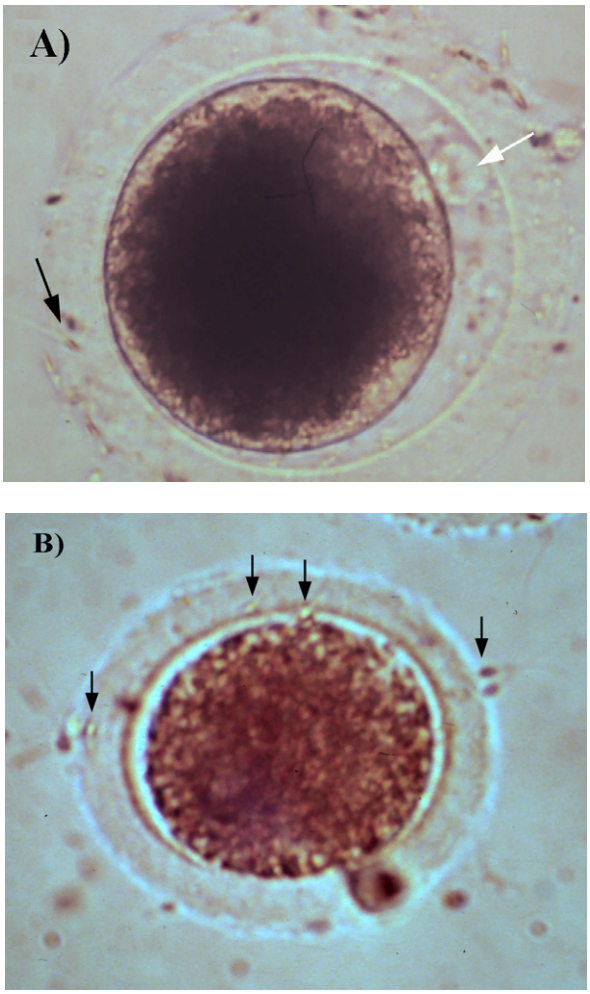

Fig. 2. Photomicrographs of African black rhinoceros oocytes. (A) MII oocyte with polar body (white arrow) and African black rhinoceros sperm penetrating the zonae pellucidae (black arrow). (B) Indian rhinoceros sperm bound and penetrating the zonae pellucidae and in the perivitelline space (black arrows).

4. Discussion

This is apparently the first report of attempted postmortem gamete rescue in the female African black rhinoceros. Although overall success was low, one embryo was produced and several oocytes achieved nuclear maturation. These initial results supported the feasibility of developing gamete rescue for this taxon, while demonstrating the need for additional research to establish a protocol that will consistently yield rhinoceros embryos.

The high number of oocytes recovered postmortem from African black rhinoceros ovaries indicated that the female rhino gonads are an excellent source of gametes for genome resource banking and assisted reproductive

procedures. The presence of fresh corpus hemorrhagicum (CH) and/or corpus albicans (CA) on the ovaries confirmed recent reproductive activity in some of the females, despite their illnesses. Therefore, it was not surprising that grades 1 and 2 oocytes were rescued from most ovaries.

Because transport time was relatively long during gamete rescue attempts, we initially thought maintaining ovaries at a cooler temperature might be beneficial. Similar gamete rescue attempts in several cat species maintained in zoos across North America have been successful with ovaries stored at 4 °C [24]. However, our initial oocyte recovery efforts with ovaries stored and transported at 4 °C did not yield any mature oocytes following 24 h IVM. The inability to mature oocytes during our early gamete rescue attempts was likely due to the negative effects of transport temperature on oocyte quality and not duration of culture, because the chromatin was degenerate. Equine oocytes mature in vitro by 24 h and simply remain arrested at MII if culture time is increased to 36 or 48 h [25]. Therefore, in subsequent gamete rescue attempts, ovaries were shipped at the recommended transport temperature for the horse (22 °C) [26] and IVM was conducted for 32 to 36 h. As a result, African black rhinoceros oocytes had distinct chromatin when stained and at least one oocyte per female achieved nuclear maturation.

That many oocytes reached MI and several underwent further development in culture, it appeared that rhinoceros oocytes can remain viable 24 to 48 h postmortem. Equine ovaries can be removed up to 5 h after euthanasia, transported up to 10 h (22 °C) and still yield oocytes that mature in culture, fertilize in vivo, and result in live offspring [26]. However, because time of death was often unknown and ovary retrieval and transport procedures were often at the discretion of each institution, further trials will be required to establish the optimum time frame for postmortem collection of rhinoceros oocytes.

Although our low success with oocyte maturation could have been due to the poor health of the individuals or the interval from animal death to time of oocyte rescue, the more likely culprits were the lack of follicle selection during oocyte collection and an imperfect culture system. Equine oocyte maturation rates varied substantially (25%–70%) [27], but even at the lower range were higher than the results achieved in the present study. Similarly, oocytes obtained by transrectal aspiration following hormonal superstimulation in the African white and black rhinoceros also had variable and low maturation rates in vitro [18]. In the present study, rhinoceros oocytes were collected from follicles that rarely exceeded 20 mm in diameter. Thus, a large proportion of oocytes were likely degenerate at the beginning of IVM. For future gamete rescues, examination of the developmental competence of oocytes from follicles of varying diameters would help establish follicle selection criteria for IVM in the African black rhinoceros. The culture protocols utilized to mature rhinoceros oocytes were based on the equine model. However, the significantly shortened LH surge and pattern of follicular development among rhinoceros species [28–31] compared with that in the horse [32–34] may have influenced the outcome of the IVM procedures. Because the in vivo maturation environment varies substantially between the rhinoceros and its closest domestic relative, further optimization of IVM procedures for the rhino taxon are warranted.

Epididymal sperm have been collected postmortem and cryopreserved in several rhinoceros species [12,15,35]. Although the functionality of cryopreserved rhinoceros semen collected via electroejaculation was demonstrated by successfully producing embryos in vivo [16,17], similar research has not yet been conducted using frozen-thawed epididymal sperm. In this study, motility of sperm declined after centrifugation to remove cryoextender and cryoprotectant in preparation for IVF. However, despite the decline in motility, sperm retained sufficient functionality in vitro to bind, penetrate, and fertilize homologous IVM oocytes. There was no difference in numbers of sperm bound either to oocytes that had been matured in culture prior to IVF, or oocytes that were inseminated immediately after recovery. Whereas the major obstacle for successful equine IVF has been the failure of sperm to penetrate the zonae pellucidae [8,36–39], this does not appear to be a problem in the rhinoceros, as both Indian and black rhinoceros sperm were found embedded in the zonae or within the perivitelline space of several black rhinoceros oocytes. That we were successful in producing a two-cell rhinoceros embryo following postmortem oocyte recovery, maturation, and fertilization using frozen-thawed epididymal sperm was noteworthy, considering only one four-cell embryo was produced after several IVF attempts with freshly collected gametes from live hormonally-stimulated African black rhinoceroses [18].

Heterologous IVF can serve as an important tool to assess the functionality of sperm in endangered species, especially when it is not feasible or practical to collect large numbers of oocytes from endangered species.

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Cross-species fertilization of oocytes from domestic species has been used successfully to examine the functional capacity of cryopreserved sperm from endangered bovids [40–42] and felids [43–45]. However, specificity of gamete interaction can be high in some species, ruling out the use of heterologous IVF systems [46]. We demonstrated herein that Indian rhinoceros sperm can bind and penetrate African black rhinoceros oocytes and confirmed the utility of gamete rescue as a means to test sperm function in the rhinoceros.

In summary, many viable oocytes were recovered from the African black rhinoceros postmortem despite lengthy delays from time of death to oocyte recovery, as long as ovaries were stored at ambient temperature. At least some of these oocytes achieved nuclear maturation in culture. Finally, homologous and heterologous frozen-thawed sperm were capable of binding, penetrating, and fertilizing African black rhinoceros oocytes in vitro. Therefore, gamete rescue offers both a means of producing rhinoceros embryos and a mechanism of testing sperm function in vitro.

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