

Technical note

Alkaline phosphatase as an indicator of true ejaculation in the rhinoceros

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Abstract

The objective was to determine if seminal alkaline phosphatase (ALP) can serve as an indicator of true ejaculation in the rhinoceros. Concentrations of ALP activity were determined in seminal fractions collected from African black rhinos (*Diceros bicornis*), an African white rhino (*Ceratotherium simum*), and an Indian rhino (*Rhinoceros unicornis*) during electroejaculation. In addition, seminal fractions collected during penile massage of a Sumatran rhino (*Dicerorhinus sumatrensis*) were assessed. Correlations between ALP activity and sperm concentration, fraction pH, and fraction osmolality were evaluated in the Indian rhino and black rhino. Concentrations of ALP activity in rhino ejaculate fractions ranged from < 5 to 11,780 U/L and were positively correlated ($P < 0.05$) with sperm concentration for both Indian rhino ($r = 0.995$) and black rhino ($r = 0.697$), but did not exhibit a strong correlation with either pH or osmolality ($P > 0.05$). Data were insufficient for establishing meaningful correlation coefficients in the Sumatran rhino and white rhino, but preliminary results were in accordance with findings in the Indian rhino and black rhino. We concluded that ALP was present in rhinoceros semen, likely originated from the epididymides and/or testes, and could serve as a useful tool for assessing the production of ejaculatory versus pre-ejaculatory fluid in the rhinoceros.

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1. Introduction

Semen collection in wildlife species is important for several reasons. First, it is often a necessary component of studies characterizing male reproductive parameters, reproductive seasonality, and the effectiveness of con-

traception. In addition, it is necessary for sperm banking and assisted reproduction. Finally, it is an essential part of male fertility examinations.

There are several reliable methods for collecting semen from most domestic species, but many of these are not routinely applicable to wildlife. Therefore, semen collection in wildlife species has often been accomplished with electroejaculation while the animal is fully anesthetized to ensure the safety of personnel and minimize stress and discomfort to the animal. Whereas

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some species respond very reliably to electroejaculation, others do not. In particular, electroejaculation has not proven very effective for semen collection in the horse [1], the domestic species most closely-related to the rhinoceros.

Semen collection by electroejaculation in the rhinoceros was first reported in 1979 [2]. Since then, improvements in methodology and equipment have made the procedure effective enough to perform reproductive studies, collect semen for assisted reproduction, and obtain high quality samples that survive cryopreservation and sperm sorting [3–9]. However, the results of electroejaculation procedures can vary substantially, even within the same animal [3,4,9]. Not only is this variability frustrating to researchers who require consistently high quality samples for their studies, but it limits the ability to conclusively assess a male rhino's potential fertility, since poor semen quality may be the result of a procedure that wasn't fully effective, rather than a true reflection of his breeding potential. Furthermore, the ability to draw conclusions regarding the impact of environmental factors (social structure, season, management style, etc.) on semen quality is hindered by the confounding factor of a procedure that inherently yields variable results. Therefore, there is a need for a diagnostic tool to conclusively determine whether an electroejaculation procedure yielded an ejaculate that accurately reflected semen quality.

Alkaline phosphatase (ALP) is present in semen of many species, and its primary site of production is the testes and epididymides in the dog [10] and horse [11]. Therefore, measurement of ALP activity can be used to confirm complete ejaculation in these species, providing a useful tool for evaluating fertility problems [11–13]. For example, ejaculates with low ALP activity indicate that full ejaculation did not occur. The problems of incomplete ejaculation and ejaculation failure have been reported in both dogs and horses [11–13], and can result from inadequate pre-copulatory stimulation or a blockage in the duct system.

If ALP is present in rhinoceros semen and originates from the epididymides or testes, it may similarly serve as a useful diagnostic tool for confirming true ejaculation in this taxon. This information is essential for properly interpreting electroejaculation results and could also be useful in assessing natural ejaculate quality and potential male rhino fertility. Therefore, the goals of this study were to determine if ALP is present in rhinoceros semen and to evaluate the potential of measuring ALP activity as an indicator of true ejaculation in this taxon.

1. Materials and methods

1.1. Animals

One Indian rhino (*Rhinoceros unicornis*), one white rhino (*Ceratotherium simum*), three black rhinos (*Diceros bicornis*), and one Sumatran rhino (*Dicerorhinus sumatrensis*) were used in this technical trial. The Indian rhino was maintained at The Wilds, Cumberland, OH. Two of the black rhinos and the white rhino were located at Busch Gardens, Tampa Bay, FL, whereas one black rhino was maintained at the Oregon Zoo, Portland, OR. The Sumatran rhino was housed at the Cincinnati Zoo & Botanical Garden, Cincinnati, OH. The 30-y-old Indian rhino contributed one ejaculate to the study, whereas the three black rhinos contributed five ejaculates, one each from a 6.5-y-old and 22-y-old rhino, and three from a rhino that was 11-, 13- and 14-y-old when the ejaculates were collected. One 9-y-old white rhino contributed one ejaculate to the study. The Sumatran rhino contributed samples on four occasions at 29 y of age. Some male rhinos are capable of siring offspring at just 4.5 y of age, but most reach sexual maturation between 5 and 7 y and continue to breed into their mid to late thirties, as long as they remain healthy and physically capable of mating. Of the rhinos in this study, five had never been given the opportunity to breed, but the Sumatran rhino and one black rhino were proven sires, and the Indian rhino's semen had produced an embryo in vivo as part of an artificial insemination project [9].

1.2. Semen collection and processing

This study was conducted opportunistically in conjunction with other studies and/or fertility examinations. For the Indian rhino, black rhinos and the white rhino, semen was collected by electroejaculation, as described in detail by Roth et al., [4]. In brief, the Indian rhino was anesthetized using a combination of M99, detomidine, and ketamine [4,9]. The black rhinos and white rhino were anesthetized using various cocktails of etorphine, detomidine, butorphanol, and ketamine, often supplemented with doxapram, midazolam and/or flunixin [4], depending on the preference of the veterinary staff at each facility. A custom-built rectal probe [4] was inserted into the rectum of the rhino and connected to an electroejaculator (P-T Electronics, Boring, OR, USA). Several series of stimulations were delivered with increasing voltage, and concurrent penile massage. Seminal fluid was collected in fractions during each of several series of stimulations, interrupted by 5 min rest periods.

Indian rhino semen was collected in multiple neat fractions (raw semen not mixed with media) into plastic collection cups and each volume was recorded. Black and white rhino semen was collected in multiple fractions into plastic bags containing 2 mL of equine semen extender (EQ) [14], modified by reducing the egg yolk concentration from 20 to 10% (“10% EQ”; supplemented with 24.0 IU/mL penicillin G and 24.3 IU/mL streptomycin sulfate; final osmolality and pH were 310–320 mOsm/kg and 7.0, respectively). Fraction volume was measured and then the 2 mL 10% EQ volume was subtracted to determine actual semen volume in the fraction. These numbers were used to calculate a dilution factor for each fraction. Sumatran rhino semen was collected in neat fractions during penile massage. For all fractions, pH was recorded and aliquots were removed to determine sperm concentration and osmolality as previously described [15]. For black rhino and white rhino samples, the sperm concentration was multiplied by the dilution factor to obtain the actual sperm concentration in each ejaculate fraction. Aliquots of each fraction were centrifuged ($1,500 \times g$) for 10 min and the supernatant recovered and stored frozen until analyzed for alkaline phosphatase.

1.3. Controls

To ensure that the 10% EQ extender in the black and white rhino samples did not contribute to the measurement of ALP in the semen, several aliquots of 10% EQ were analyzed for ALP. These controls included: 1) neat 10% EQ; 2) 10% EQ diluted 1:2 with a Tyrode’s salt solution containing BSA (0.6% (v/v); Sigma A-4697) lactate, pyruvate, HEPES, 125 IU/mL penicillin-G and 38 IU/mL streptomycin sulfate (HEPES-TALP; modified from Parrish *et al.* [16]; and 3) 10% EQ diluted 2:1 with TALP. In addition, to ensure EQ did not interfere with our ability to measure ALP in semen, a neat semen sample was analyzed for ALP then diluted 1:1 and 1:2 with 10% EQ and analyzed again for ALP. Finally, since some samples were frozen and thawed twice before analysis, ALP was measured in a sample thawed once, and three times to determine if the activity diminished with multiple freeze-thaw cycles.

1.4. Alkaline phosphatase analysis

Alkaline phosphatase (p-nitro-phenylphosphate) activity in each sample was determined using an Olympus Analyzer (Olympus AU400E, Olympus America Inc., Melville, NY, USA) with Olympus reagents and standards. The analyzer was calibrated daily using Olympus calibrators according to the manufacturer’s instruc-

tions, and assay performance was monitored by analyzing two dilutions of commercially-available external controls (Bio-Rad Laboratories, Hercules, CA, USA) and two dilutions of internal controls. All tests were performed at 37 °C. The ALP value produced by the analysis was multiplied by the appropriate dilution factor for any sample containing EQ to calculate the accurate concentration of ALP activity in the original semen fraction.

1.5. Statistical analysis

All data were entered into the Macintosh Statview software program and Indian rhino and black rhino data sets were analyzed using the Spearman Rank Order Correlation Test to determine relationships between sperm concentration and ALP activity, pH, and osmolality. For all analyses, $P < 0.05$ was considered significant. Data were also entered into the Microsoft Office Excel 2007 software program, and \log_{10} scale graphs depicting the relationship of ALP activity to sperm concentrations were produced for Indian rhino and black rhino samples. Linear, quadratic and power trend lines were calculated by the Microsoft Office Excel software program for each data set and that with the highest R^2 value was included on each graph.

2. Results

2.1. Rhino samples

All Indian rhino, African black rhino and African white rhino seminal fractions in the study ($n = 11$, $n = 15$ and $n = 3$, respectively) contained sperm, but sperm concentration in each fraction varied considerably, ranging from 3 to $2,080 \times 10^6/\text{mL}$, 2.3 to $484 \times 10^6/\text{mL}$, and 6 to $49 \times 10^6/\text{mL}$ in the three species, respectively. Similarly, concentrations of ALP activity varied notably between fractions of each species, ranging from 11 to 3,784 U/L, 54 to 11,780 U/L and 17 to 52 U/L, respectively. Fraction pH ranged from 7.5 to 8.7, 7.3 to 8.0, and 7.5 to 7.9 for Indian, black and white rhino samples, respectively, and osmolality ranged from 266 to 299 mOsmol/kg, 291 to 336 mOsmol/kg, and 316 to 321 mOsmol/kg, respectively.

A total of seven Sumatran rhino seminal fractions collected by manual massage were analyzed in the study. Two of these samples contained sperm at concentrations of 1 and $38 \times 10^6/\text{mL}$, but no sperm were motile. Fraction pH and osmolality varied from 8.3 to 9.0 and 272 to 435 mOsmol/kg, respectively.

For the Indian rhino and African black rhino data sets there was a strong, positive correlation between

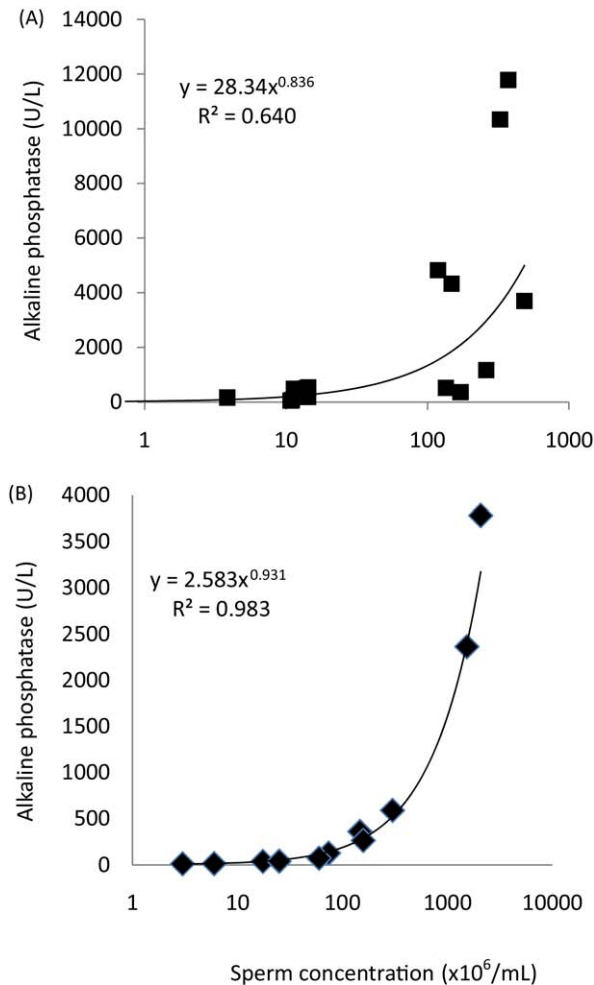


Fig. 1. Alkaline phosphatase activity and sperm concentrations in seminal fractions collected from three African black rhinos (A; $n = 15$ fractions) and an Indian rhino (B; $n = 11$ fractions) during electroejaculation. The power trendline fit the data of both species best.

ALP activity and sperm concentrations in seminal fractions (Fig. 1; $r = 0.995$ and 0.697 , respectively; $P < 0.05$). In contrast, there was a negative correlation between pH and ALP activity, but it was weak ($P > 0.05$) in both Indian rhino ($r = -0.242$) and black rhino ($r = -0.510$). The relationship of osmolality and ALP activity varied from a weak positive correlation in the black rhino ($r = 0.201$) to a weak negative correlation in the Indian rhino ($r = -0.199$). The power trend line best fit the data sets of both black rhinos ($R^2 = 0.640$; Fig. 1A) and Indian rhinos ($R^2 = 0.983$; Fig. 1B). Due to the small number of samples available for the study following electroejaculation in the white rhino ($n = 3$), data for this species were considered preliminary only and insufficient for calculating correlation coefficients.

None of the Sumatran rhino fractions collected by manual massage contained measurable ALP activity (all < 5 U/L).

2.2. Control samples

The ALP activity in the neat 10% EQ control sample was barely detectable at 8 U/L, whereas ALP activity in the 10% EQ controls diluted 2:1 or 1:2 (v:v) with TALP was undetectable (< 5 U/L). A neat ejaculate fraction initially containing 3784 U/L ALP was thawed twice more and then analyzed again for ALP. This second analysis resulted in an ALP activity value of 4292 U/L. When the same aliquot was analyzed for ALP after a 1:1 and 1:2 (v:v) dilution with 10% EQ the concentrations of activity were 2112 U/L and 1065 U/L, respectively.

3. Discussion

This paper is the first report of ALP in rhinoceros semen and provides evidence for a direct, positive correlation between ALP and ejaculate sperm concentrations in the Indian rhino and African black rhino, as well as preliminary data in the Sumatran rhino and African white rhino. These data supported the suggestion that ALP is produced primarily in the epididymides and/or testes of the rhinoceros and can be used as a diagnostic tool for distinguishing ejaculatory from pre-ejaculatory fluid in this species. In contrast, the relationships between osmolality or pH and sperm concentrations were variable and weak, and therefore not useful in identifying fractions of ejaculatory fluid.

Similar to reports in other species, the concentrations of ALP activity in rhino seminal fractions varied extensively from < 5 U/L to $> 11,000$ U/L but were somewhat lower than the high values reported in the horse (48,700 U/L) [11], dog (40,000 U/L) [17] and beluga whale ($> 30,000$ U/L) [18]. Perhaps the difference is an artifact of electroejaculation, since the latter three species were collected naturally into an artificial vagina or by manual massage. Alternatively, the lower values could be a product of the small sample size in this study, since there was wide inter- and intra-individual ranges of normal ALP activity, and the fertility of some of the rhinos was unknown. However, it is also possible that the rhinoceros produces lower seminal ALP activity compared to these other species. Very high sperm concentrations ($2,080 \times 10^6$ sperm/mL) in some fractions refuted the possibility that all samples were primarily composed of pre-ejaculatory fluid and therefore contained lower ALP activity.

Seminal ALP activity in complete ejaculates varies among species, even between those primarily producing ALP in the epididymides and testes region. For example, in the horse, ALP activity < 100 U/L is associated with pre-ejaculate fluid, whereas ALP activity > 1,000 U/L is indicative of complete ejaculation [11]. In contrast, ALP activity < 5,000 U/L is indicative of incomplete ejaculation in the dog [12]. Based on our preliminary data, it appears that the rhino may be similar to the horse with seminal ALP activity < 100 U/L associated with pre-ejaculatory fluid, and seminal ALP activity > 1,000 U/L indicative of samples containing considerable contributions from the epididymides and/or testes. However, species differences may exist within the taxon, and a more robust data set would be required to determine specific ALP values for each rhino species. It is worth noting that seminal fractions with low ALP activity (< 100 U/L) can still contain relatively useful sperm concentrations for reproductive studies and assisted reproduction (10 to 50×10^6 /mL in black rhino, 17 to 60×10^6 /mL in the Indian rhino, and 9 to 49×10^6 /mL in the white rhino).

Since this study was conducted opportunistically in conjunction with several other projects, several control samples had to be analyzed to ensure that the data truly reflected ALP production by the male rhino and not an artifact of any processing methodology used in association with the other research projects. The data were convincing in demonstrating that EQ or multiple freeze-thaw cycles did not interfere with the ability to accurately assess ALP activity.

Data for the Sumatran rhino and African white rhino were minimal and very preliminary. However, the low ALP activity in most of the Sumatran rhino samples suggested that the fluid collected during penile massage was pre-ejaculatory fluid, and the sperm in some of those samples were presumptively old, residual cells flushed from the tract, rather than the product of active ejaculation during the procedure. This Sumatran rhino had sired three calves, many of his natural ejaculates had been collected from the female post-mating, and all of those samples contained motile sperm [15]. Likewise, the few white rhino samples analyzed herein contained relatively low sperm concentrations, suggesting they represented only pre- or partial ejaculate fractions. The very high sperm concentrations ($> 1,000 \times 10^6$ sperm/mL) produced in some fractions of the Indian rhino ejaculate were not surprising, as this species is known to produce higher sperm concentrations than the other rhino species [9,19]. Even with ALP activity three times as high as any measured in the Indian rhino

fractions, the black rhino fractions never contained $> 500 \times 10^6$ sperm/mL.

In conclusion, ALP was present in rhinoceros semen and its positive correlation with sperm concentration indicated that it originated from the epididymides and/or testes. Therefore, measuring ALP activity in semen samples collected from the rhinoceros should provide an easy diagnostic tool for distinguishing ejaculatory fluid from pre-ejaculatory fluid in most cases. Furthermore, if ALP production persists in the face of declining testicular sperm production, then ALP activity in rhinoceros semen samples that contain few or no sperm could aid in determining if that sample represents that rhino's true semen quality (high ALP activity), or if it is merely reflecting incomplete ejaculation or ejaculation failure (low ALP activity).

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