Feasibility of sex-sorting sperm from the white and the black rhinoceros (*Ceratotherium simum, Diceros bicornis*)

B. Behr [a,*], D. Rath [b], P. Mueller [c], T.B. Hildebrandt [a], F. Goeritz [a], B.C. Braun [a], T. Leahy [d], S.P. de Graaf [d], W.M.C. Maxwell [d], R. Hermes [a]

[a] *Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany*
[b] *Institute of Farm Animal Genetics, Friedrich-Loeffler-Institute, Federal Institute for Animal Health, Mariensee, Germany*
[c] *Department of Biology, Molecular Biophysics, Humboldt University of Berlin, Berlin, Germany*
[d] *Faculty of Veterinary Science, University of Sydney, Sydney, NSW, Australia*

Abstract

The objective of these studies was to investigate the practicality of flow cytometric sex-sorting for spermatozoa from the white and the black rhinoceros (*Ceratotherium simum, Diceros bicornis*). In Experiment 1, four semen extenders were tested regarding their suitability for liquid preservation of spermatozoa before sorting. Dilution in MES-HEPES–based semen extender followed by incubation generated best sperm quality parameters (motility, viability, and acrosome integrity). In Experiment 2, the effect of staining method (15 °C for 4 to 6 h during transport or 37 °C for 1 to 1.5 h) on sort efficiency and sperm quality was investigated. Staining at 15 °C during transport resulted in a higher percentage of sperm samples showing a resolution of X- and Y-chromosome–bearing populations (60%) compared with that for staining at 37 °C after transport (33%) and resulted in superior sperm integrity after staining (43.8 ± 11.3% vs. 19.6 ± 12.1%). Sort rate was 300 to 700 cells/sec and sort purity, determined for one sorted sample, was 94% for X-chromosome–bearing spermatozoa. In Experiment 3, the highly viscous component of rhinoceros seminal plasma, which complicates the process of sperm sorting, was examined by gel electrophoresis and mass spectrometry. Results suggested a 250-kDa glycoprotein (most likely originating from the bulbourethral gland) to be responsible for the characteristic viscosity of ejaculates. In Experiment 4, viscosity of seminal plasma, as measured by electron spin resonance spectroscopy, was significantly decreased after addition of α-amylase or collagenase (0.5 and 3 IU per 100 μL seminal plasma, respectively) by 28% and 21%, respectively, with no negative effect on sperm characteristics. The results of this study demonstrate for the first time that rhinoceros spermatozoa can be successfully sorted into high-purity X- and Y-chromosome–bearing populations. Furthermore, the successful liquefaction of viscous ejaculates provides the means to greatly improve sort-efficiency in this species.
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1. Introduction

Sex sorting of spermatozoa by modified flow cytometry has been applied to a range of species, and numerous offspring were born after artificial insemination (AI), in vitro fertilization (IVF), and intracytoplasmic sperm injection (ICSI) with sorted spermatozoa [1]. In exotic and endangered species such as the rhinoceros, the

* Corresponding author. Tel.: +89 2180 78864; fax: +89 2180 78801.
E-mail address: b.behr@med.vetmed.uni-muenchen.de (B. Behr).

possibility of producing a larger number of females and hence accelerating population growth makes the implementation of sex-sorting technology particularly appealing [2,3]. In addition, sex-preselection by sperm sorting has the potential to offset breeding losses from premature female aging [4] and to balance skewed sex ratios in the black [5,6] and Indian rhinoceros [7,8].

Semen from rhinoceroses has been routinely collected by electroejaculation [9,10], successfully cryopreserved [9,11], and artificially inseminated either fresh or frozen [3,12]. Whereas a theoretical sex-sorting index for rhinoceros spermatozoa has been established [13], the development of a sperm-sorting protocol for this species remains in its infancy. Spermatozoa of domestic species such as sheep and cattle have benefited from almost two decades of sex-sorting protocol optimization and diluent development in an effort to maximize protection against the myriad of stressors to which they are exposed [14,15]. Sperm physiology is partly species-specific, and often the available knowledge is very limited or absent in wildlife species, resulting in the need to develop new protocols for sperm handling prior, during, and after the sorting process [16].

A major challenge in handling and processing ejaculates from African rhinoceroses for flow cytometric sex-sorting, especially those from the white rhinoceros, is the high viscosity of semen samples. Upon ejaculation, spermatozoa are mixed with secretions from the accessory sex glands, entrapping the spermatozoa in a highly viscous gel (unpublished observations). Spermatozoa cannot be separated from the gelatinous material by filtration or centrifugation as routinely applied in the boar or the stallion, nor does the gel fraction liquefy after a short period of time (human [17]) or release spermatozoa by contraction (rhesus monkey [18]). These limitations are also evident in the semen samples of camelids such as the alpaca and llama [19,20].

The main source of coagulating substrates in most mammals, including the human, is the vesicular glands [21]. The African rhinoceros has three accessory sex glands, which may contribute to the high viscosity of seminal plasma: (i) the paired long-shaped bulbourethral glands, (ii) the prostate, and (iii) the paired vesicular glands [9,22].

A number of different methods have been explored to characterize and reduce the viscosity of seminal plasma in several mammalian species. In the human, a chymotrypsin-like prostatic-specific antigen was determined to cause liquefaction of semen through cleavage of semenogelin I, the major component of the coagulum, which is expressed exclusively in the vesicular glands [23–25]. Additionally, proteolytic enzymes were

detected and isolated in the seminal plasma of different species such as collagenase-like metalloproteinases in human, rat, and bull semen [26] and trypsin in semen from the guinea pig and rabbit [27,28]. Based on these findings, several enzymes have been used to cleave the gelatinous components of seminal plasma in order to reduce its viscosity. Trypsin and collagenase have been used as additives to reduce the viscosity of monkey [29], guinea pig [27] and, more recently, alpaca and llama ejaculates [30,31]. While reducing the viscosity of seminal plasma, Bravo and co-workers claimed that collagenase had no impact on functional parameters of spermatozoa in the alpaca and llama [30], and no effect on cleavage and embryo development rates after IVF in llamas was observed [32]. The glycoside hydrolase enzyme α-amylase was successfully used to liquefy coagulated human semen [33] but failed to reduce the viscosity of guinea pig or rhesus monkey semen [29]. In these investigations, the influence of amylase on sperm fertility was not determined. However, a study on bull semen extenders showed that the addition of α-amylase to bull spermatozoa had a significantly positive effect on pregnancy rates after AI [34].

The aim of the current study was twofold. We first attempted to determine optimal techniques for semen extension during chilled transport and staining of spermatozoa preliminary to sex sorting (Experiments 1 and 2). Second, we tried to investigate the origin and composition of the gelatinous fraction in the rhinoceros seminal plasma, with the ultimate goal of enzymatic liquefaction of this fraction without compromising the functional integrity of spermatozoa (Experiments 3 and 4).

2. Materials and methods

Nineteen semen samples from 13 white rhinoceros (WR; *Ceratotherium simum*) bulls and seven ejaculates from four black rhinoceros (BR; *Diceros bicornis*) bulls were collected using electroejaculation under general anesthesia [9]. Unless otherwise mentioned, all materials were of reagent grade or higher and were purchased from Sigma Aldrich Chemie GmbH (Taufkirchen, Germany).

2.1. Experiment 1: Effect of extender on quality of chilled rhinoceros spermatozoa

Seven semen samples from 7 WR bulls were used for this experiment. After semen collection, aliquots were immediately diluted 1:1 (v:v) with four different, prewarmed (37 °C) semen extenders: (1) Blottner's sorting medium (BSM), a MES[2-(N-morpholi-

no)ethane sulfonic acid]-HEPES [N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid] and skim milk–based medium [35]; (2) MES-HEPES semen extender (MES) [36]; (3) Andromed (Minitüb, Tiefenbach, Germany); (4) Beltsville thawing solution (BTS) [37]. All extenders were supplemented with sodium pyruvate (1.0 mM; as an antioxidant) and EGTA (ethylene glycol bis 2 aminoethyl ether; 2.0 mM; to prevent sperm agglutination). Smears from each sample were prepared for assessment of morphology and viability of spermatozoa. Samples were then chilled slowly during transport by placing the test tube with the semen inside an isothermal water bath stored at 4 °C. Chilled samples were transported to the laboratory over 4 to 10 h, incubated at room temperature (RT) for 30 min then at 37 °C for 15 min before evaluating sperm-quality parameters. These included total motility (TM), progressive motility (PM), viability, and acrosome integrity of spermatozoa.

2.1.1. Assessment of spermatozoa

Motility, viability, and morphology of spermatozoa were evaluated after collection, transport to the laboratory, DNA staining and incubation, and sex sorting. Progressive motility and TM of spermatozoa were determined as described by Hermes et al. [9]. Evaluation of sperm motility was complicated by high viscosity of semen samples. As viscosity of semen samples was not homogenous, it was attempted to evaluate motility parameters in sample parts of preferably low viscosity. The viability and acrosome integrity of spermatozoa were assessed using an adapted Kovács-Foote staining technique [38,39]. Sperm sample aliquots (10 µL) were diluted 1:5 (v:v) with phosphate-buffered saline solution (PBS). Ten-microliter diluted samples were stained with 10 µL of an 0.16% Chicago Sky Blue solution (diluted in PBS) by mixing the drops on a slide before making smears. The smears were fixed for 2 min in a fixative composed of 86 mL 1.0 N HCL plus 14 mL formaldehyde solution (37% wt/wt) and 0.2 g neutral red. The acrosome stain was a 7.5% Giemsa stock solution (GS-500) diluted in distilled water. Smears were stained for 5 h at 35 °C. A total of 200 stained spermatozoa were counted in duplicate using oil immersion and at ×1000 magnification on a standard bright-field microscope. Spermatozoa were categorized as "viable" (head membrane intact; posterior part of the sperm head white to light blue) or "dead" (head membrane damaged; posterior part of the sperm head dark blue). Acrosomes were classified as intact (anterior part of the sperm head pink to purple), modified (anterior part of the sperm head dark

lavender), or reacted (anterior part of the sperm head light lavender) including completely detached acrosomes (anterior part of the sperm head white to gray).

2.2. Experiment 2: Effect of DNA staining method on sort efficiency and post-sort quality of rhinoceros spermatozoa

Only spermatozoa extended in MES extender were subjected to DNA staining. Aliquots from 10 ejaculates were stained after chilled transport (4 °C; 4 to 10 h) to the laboratory at 37 °C for 1 to 1.5 h after rewarming to RT for 30 min (10 WR). Spermatozoa from 10 different ejaculates were stained during transport for 4 to 6 h at 15 °C (7 WR, 3 BR). For DNA staining at 37 °C, only spermatozoa with a TM ≥35% after transport were used. Aliquots of 0.5 mL (sperm concentration: 50×10^6 cells/0.5 mL) from each treatment were labeled with Hoechst 33342 DNA stain at three different concentrations (7.5, 10, and 12.5 µL of 26.7 mM stock solution). Stained spermatozoa were assessed as described in Section 2.1.1 and sorted on a high-speed flow cytometer (MoFlo SX; Dako Colorado, Fort Collins, CO, USA) equipped with an argon laser (200 mW) and operating at 40 psi with a HEPES buffer–based sheath fluid [40]. Sortability of labeled samples was estimated in the flow cytometer through optic purity of the resolution between X- and Y-chromosome–bearing sperm populations, visible in the dot plot output. Samples with good resolution between the X- and Y-chromosome–bearing spermatozoa were sorted into two 10-mL conical plastic tubes (Greiner, Nürtingen, Germany) containing 500 µL TEST-yolk collection extender, a TES/tris-based medium, with an egg yolk concentration of 2% [41]. After sorting, samples were centrifuged at $840 \times g$ at room temperature for 20 min to remove the sheath fluid. The supernatant was discarded, and the pellet was resuspended in 100 µL of Blottner's cryomedia semen extender [9,36]. An aliquot of the X- and Y-sorted sample from one bull was restained to determine the proportions of X- and Y-chromosome-bearing spermatozoa [42].

2.3. Experiment 3: Characterization of viscous seminal plasma fraction

2.3.1. Rating and preparation of seminal plasma and secretions of the accessory sex glands

The viscosity of collected semen was macroscopically evaluated based on the ability to raise a 10 µL semen aliquot in a 10- to 100-µL pipette (ABIMED, Langenfeld, Germany). Viscosity was subjectively divided into three groups: I, not viscous (unhindered

filling); II, viscous (decelerated filling); and III, highly viscous (filling not feasible). Seminal plasma aliquots from 10 ejaculates (6 WR, 4 BR) were stored for further assessment. When possible, seminal plasma was separated from spermatozoa by centrifugation for 20 min at $1000 \times g$. The supernatants were transferred into 1.5-mL tubes, recentrifuged for 25 min at $4000 \times g$ to eliminate the remaining cells, and stored at $-80\ ^{\circ}C$. When separation of spermatozoa and seminal plasma was not possible due to high viscosity, aliquots of semen were stored at $-80\ ^{\circ}C$ without treatment. Additionally, secretions of postmortem-extracted bulbourethral glands, prostate and vesicular glands from two WR bulls were ejected and stored at $-80\ ^{\circ}C$.

2.3.2. Gel electrophoresis

All reagents and apparatus used in the preparation and separation of the protein samples were from Bio-Rad (Hercules, CA, USA) unless otherwise specified. Thawed seminal plasma aliquots were centrifuged at $10,000 \times g$ for 10 min to remove cellular components. Proteins of seminal plasma samples and secretions from the bulbourethral glands, prostate and vesicular glands were comparatively separated by one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) after determination of quantitative protein content using bicinchoninic acid [43]. Samples were subjected to SDS-PAGE as described by Laemmli [44] using a 12% polyacrylamide gel. Twenty-microliter samples containing 28 μg total protein were mounted to the gel. Molecular mass was estimated by comparison with Page Ruler Unstained Protein Ladder standards (10 μL) or the Precision Plus Protein Standard (3 μL). Electrophoresis was run at 120 V for 90 min. After electrophoresis, gels were stained with Coomassie Blue G250 for 2 h and washed for 1 h in 10% ethanol (vol/vol) and 2% ortho-phosphoric acid (vol/vol).

For further characterization of seminal plasma proteins, two-dimensional gel electrophoresis (2-DE) was performed. Protein samples were mixed with a 2-DE–compatible buffer containing 5 M urea, 2 M thiourea, 2% (wt/vol) CHAPS (3-[(3-Cholamidopropyl)-dimethylammonio]-1 propanesulfate, 40 mM Tris–HCl, 2 mM TBP (tributylphosphine), 2% (wt/vol) sulfobetaine 3–10, 1% (vol/vol) Bio-Lyte 3–10 carrier ampholytes, and 0.002% (wt/vol) bromophenol blue, followed by centrifugation ($16,000 \times g$, 1 min, $25\ ^{\circ}C$) to remove insoluble material. Samples (100 μg protein, 250 μL) were loaded onto an 11-cm pH 3–10 linear pH gradient IPG (inositolphosphate glycan) gel strip. After overnight in-gel rehydration, isoelectric focusing was carried out by a IEF (isoelectric focussing) cell apparatus using a

five-step program (2 h at 200 V, 2 h at 500 V, 2 h at 1 kV, 2 h at 3 kV, and 50 kVh at 5 kV) for a total of 55 to 60 kVh. After focusing, the IPG strips were equilibrated for 10 min in 6 M urea, 2% (wt/vol) SDS, 20% (vol/vol) glycerol, 5 mM TBP, 2.5% (vol/vol) acrylamide, and 350 mM Tris–HCl (pH 8.8), then transferred onto a second dimension sodium SDS-PAGE gel (4% to 12% linear gradient) and sealed in place with 0.5% (wt/vol) agarose in MES buffer with a trace of bromophenol blue. Second-dimension electrophoresis was carried out using a Criterion system at 180 V for 55 min in MES buffer. Gels were stained with Sypro Ruby overnight and washed in 10% (vol/vol) methanol and 7% (vol/vol) acetic acid for 1 h before being visualized on a Molecular Imager FX Pro Plus. Gels were post-stained with Coomassie Blue G250 as described above.

2.3.3. In-gel digestion and mass spectrometry

Selected bands displayed in the one-dimensional SDS-PAGE from seminal plasma and accessory sex gland secretions were analyzed using mass spectrometry [45]. Briefly, selected protein bands were destained, the gel pieces then dried in a rotor evaporator and rehydrated with 15 μL porcine sequencing grade trypsin at 125 ng/μL. After 1 h at $4\ ^{\circ}C$, any excess trypsin was removed and 15 μL 20 mM ammonium bicarbonate solution added and the samples incubated at $37\ ^{\circ}C$ overnight. For matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis, 1 μL of sample was spotted onto a target plate with an equal volume of matrix solution and air-dried at room temperature. Mass spectra were acquired in the mass-charge range of 800 to 3500 m/z on a QSTAR XL mass spectrometer equipped with a MALDI source (Applied Biosystems Inc., Foster City, CA, USA). Mass calibration was performed using fibrinopeptide B as an external calibrant. The generated monoisotopic peak masses were subjected to database searching against the MSDB (Microsoft System Database) comprehensive nonredundant database using MASCOT version 2.0 (Matrix Science, London, UK). The peptide mass fingerprinting (PMF) data were also searched using the MS-Fit program (Protein-Prospector, UCSF Mass Spectrometry Facility). Each PMF spectrum was manually inspected, and peptides were obtained by manual annotation of the spectra. Parameters for protein identification included searching with a mass error tolerance of 65 ppm per peptide, 1 missed tryptic cleavage, and allowing oxidation of methionine as an optional modification. No isoelectric point (Ip) or mass restrictions were included. Confident matches were defined by the MASCOT score and statistical significance ($P < 0.05$), the number of match-

ing peptides, and the percentage of total amino acid sequence covered by those matching peptides.

2.4. Experiment 4: Liquefaction of viscous fraction using α-amylase and collagenase and evaluation of the enzyme influence on sperm characteristics

2.4.1. Electron spin resonance spectroscopy

α-Amylase 0.5 IU and collagenase 3 IU dissolved in MES semen extender (1 mg/mL) were mixed with 100-μL aliquots of undiluted seminal plasma from 7 WR bulls. In this experiment, MES semen extender was used instead of PBS for dissolving the enzymes to avoid a possible damaging influence of diluent medium on sperm quality with regard to an enzyme use with native semen samples. As a control, 100-μL samples of undiluted seminal plasma were mixed with 10 μL MES semen extender. Samples were incubated for 30 min at RT. The viscosity of enzyme-treated and control seminal plasma samples were characterized by using electron-spin resonance spectroscopy (ESR) to measure the rotation of the paramagnetic substance 2,2,6,6-tetramethyl-1-piperidinyloxy (Tempo) [46]. This assay has the advantage of allowing measurement of sample volumes as low as 50 μL. The rotation of Tempo in aqueous solution depends on the solution viscosity and can be estimated from the shape of the ESR spectrum. Five microliters of a 1 mM Tempo solution was added to the seminal plasma samples incubated for 30 min at RT. Subsequently, ESR spectra were recorded at 4 °C on a Bruker ECS 106 spectrometer (Bruker, Karlsruhe, Germany) with the following settings: modulation amplitude, 1 G; power, 10 mW; scan width, 100 G; accumulation, once. To quantify solution viscosity, a rotational correlation time (τ_c) was estimated from the ESR spectra [47,48].

2.4.2. SDS-PAGE

SDS-PAGE was used to evaluate the effect of α-amylase (31.8 IU/mg) and collagenase (196 IU/mg) on the protein profile of rhino seminal plasma. Three enzyme concentrations (α-amylase: 3 IU, 1.5 IU, 0.5 IU; collagenase: 18.5 IU, 9.25 IU, 3 IU) diluted in 10 μL PBS were added to the seminal plasma sample mounted to the gel. In the control sample, only 10 μL PBS was added. After 30 min incubation at RT, samples were subjected to the SDS-PAGE as described earlier.

2.4.3. Quality parameters of spermatozoa after treatment with α-amylase and collagenase

Directly after collection, aliquots from ejaculates were diluted 1:1 (v:v) in MES semen extender. Three concentrations of enzymes (α-amylase [n = 12; 8 WR, 4 BR]: 0.16 IU, 1.6 IU, 4 IU; collagenase [n = 8; 4 WR, 4 BR]: 24.5 IU, 9.8 IU, 0.98 IU) dissolved in prewarmed (37 °C) MES semen extender (1 mg/mL, 5, 50, 125 μL of enzyme solution) were added to 0.5-mL semen samples. As a control, 125 μL MES semen extender without any enzyme addition was added to 0.5-mL semen aliquots. Sperm PM and TM, viability and acrosome integrity were evaluated after dilution and 1 h after addition of the enzyme, respectively.

2.5. Statistical analyses

Statistical analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Due to small number of samples, nonparametric tests were performed. Differences in viscosity and quality parameters of spermatozoa between enzyme-treated and control groups as well as between semen samples diluted in different extenders after transport to the laboratory were analyzed using rank variance analysis (exact Friedman test). A multiple paired comparison (Wilcoxon and Wilcox test [49]) was used to further define differences between the groups. Quality parameters of spermatozoa were compared between samples after collection, transport, and after staining and incubation with Wilcoxon signed rank test with continuity correction or exact Mann-Whitney U-test. For all analyses, the level of significance was set to $P \leq 0.05$.

3. Results

The average concentration of spermatozoa in collected ejaculates was $248.0 \pm 470.2 \times 10^6$ spermatozoa/mL (mean ± SD; n = 26). The median concentration was 80×10^6 spermatozoa/mL. Two from 26 values were defined as extremes in the boxplot option (1500×10^6 and 2000×10^6 spermatozoa/mL; data not shown). Mean sperm concentration ± SD excluding these extremes was $117.6 \pm 112.3 \times 10^6$ spermatozoa/mL. The mean volume of ejaculates collected was 20.8 ± 15.7 mL. Progressive motility, TM, and sperm integrity (viable, intact acrosome) were $38.5 \pm 24.4\%$, $73.5 \pm 16.0\%$, and $73.3 \pm 12.0\%$, respectively. The high viscosity of the seminal fluids in most ejaculates made the evaluation of PM and TM difficult, as most spermatozoa were "trapped" in the gel fraction.

3.1. Experiment 1: Effect of extender on quality of chilled rhinoceros spermatozoa

The quality parameters of spermatozoa after collection and after transport are presented in Table 1.

Table 1
Sperm-quality characteristics (mean ± SEM) after collection and after transport to the laboratory (4 to 10 h, 4 °C), comparatively displayed after dilution in four semen extenders (MES, Andromed, BTS, BSM; n = 7).

Extender	Quality parameter	After collection (%)	After transport (%)
MES	Progressive motility	57.3 ± 4.2^a	$19.4 \pm 7.1^{b,c}$
	Total motility	74.9 ± 5.2^a	$56.9 \pm 6.9^{b,c}$
	Intact[*]	68.3 ± 7.1	53.4 ± 9.3
	Viable	74.1 ± 5.9	61.9 ± 8.4
	Intact acrosomes	70.1 ± 6.9	54.7 ± 9.6
Andromed	Progressive motility	37.6 ± 11.2^a	$3.6 \pm 1.8^{b,d}$
	Total motility	68.1 ± 8.7^a	$34.6 \pm 9.4^{b,d}$
	Intact[*]	69.0 ± 8.3	50.1 ± 6.4
	Viable	75.0 ± 7.5	62.6 ± 6.1
	Intact acrosomes	71.6 ± 8.3	52.9 ± 6.8
BTS	Progressive motility	53.1 ± 10.8^a	14.1 ± 7.9^b
	Total motility	71.1 ± 8.2^a	51.6 ± 10.9^b
	Intact[*]	68.7 ± 7.5^a	51.3 ± 23.6^b
	Viable	77.3 ± 5.9	64.1 ± 8.7
	Intact acrosomes	71.1 ± 7.9	50.7 ± 9.4
BSM	Progressive motility	35.0 ± 10.7	14.7 ± 8.5
	Total motility	60.0 ± 9.5	47.0 ± 10.4
	Intact[*]	69.6 ± 7.5^a	53.5 ± 7.5^b
	Viable	77.0 ± 6.0	64.7 ± 7.1
	Intact acrosomes	71.7 ± 7.6	54.8 ± 7.6

[*] Intact = viable, intact acrosome.[a–d] Within lines (a,b) and columns (c,d), values of same sperm parameter with different superscripts differ significantly ($P \leq 0.05$).

After transport to the laboratory, semen samples extended in MES had a significantly higher PM and TM than did those in Andromed (Wilcoxon and Wilcox test: PM, df = 3, $P \leq 0.01$; TM, df = 3, $P \leq 0.05$). Progressive motility and TM were also higher in MES than in BTS and BSM, but these differences were not significant. There was a decrease of the quality parameters of spermatozoa during transport to the laboratory regardless of the extender used, even if not

all declines were significant (Wilcoxon signed rank test with continuity correction).

3.2. Experiment 2: Effect of DNA staining method on sort efficiency and post-sort quality of rhinoceros

The quality parameters of spermatozoa after staining and incubation are presented in Table 2 (n = 10 [15 °C/ 37 °C]).

Table 2
Sperm-quality characteristics (mean ± SEM) comparatively displayed after collection/dilution in MES semen extender, after staining and incubation at 37 °C or 15 °C [n = 10 (15 °C/37 °C)], and after sex sorting [n = 4 (15 °C); n = 3 (37 °C)].

Quality parameter	After collection (%)	After incubation at 37 °C (%)	After sorting[†] (%)
Total motility	70.6 ± 6.4^a	$13.3 \pm 6.9^{b,c}$	11.7 ± 7.6
Intact[*]	67.7 ± 4.5^a	$19.6 \pm 3.8^{b,c}$	30.0 ± 8.2
Viable	72.5 ± 3.7^a	$28.5 \pm 4.9^{b,c}$	46.3 ± 12.9
Intact acrosome	70.4 ± 4.5^a	$21.3 \pm 3.6^{b,c}$	33.3 ± 8.3
Quality parameter	After collection (%)	After incubation at 15 °C (%)	After sorting[†] (%)
Total motility	72.7 ± 3.0^a	$32.2 \pm 4.1^{b,d}$	11.5 ± 6.1
Intact[*]	74.6 ± 2.0^a	$43.8 \pm 3.6^{b,d}$	37.7 ± 6.7
Viable	78.5 ± 2.1^a	$49.9 \pm 3.7^{b,d}$	49.3 ± 8.4
Intact acrosome	76.1 ± 1.9^a	$46.4 \pm 3.5^{b,d}$	42.0 ± 5.4

[*] Intact = viable, intact acrosome.
[†] Due to small number of samples (n = 3, 37 °C; n = 4, 15 °C), no statistical testing was performed.[a–d] Within lines (a,b) and columns (c,d), respectively, values of same sperm parameter with different superscripts differ ($P < 0.05$).

Table 3
Sperm quality characteristics (mean ± SEM) comparatively displayed after dilution in MES semen extender and after addition of 16 IU α-amylase (A; n = 12) and 24.5 IU collagenase (C; n = 8) to 0.5-mL semen aliquots and incubation for 1 h at RT (T1). Controls were incubated for 1 h at RT after addition of 125 μL MES.

Quality parameter	After dilution (A) (%)	T1 (A) (%)	T1 control (A) (%)	After dilution (C) (%)	T1 (C) (%)	T1 control (C) (%)
Progressive motility	51.3 ± 7.3	54.4 ± 7.9	54.2 ± 7.5	43.1 ± 8.8	46.6 ± 9.4	51.8 ± 10.0
Total motility	78.5 ± 4.0	71.4 ± 6.0	73.5 ± 6.2	75.6 ± 5.2	66.9 ± 7.8	69.2 ± 8.4
Intact*	73.5 ± 3.3	74.0 ± 4.0	71.9 ± 3.8	70.0 ± 4.7	70.6 ± 3.9	71.8 ± 5.2
Viable	78.4 ± 2.8	78.9 ± 2.9	77.5 ± 3.1	75.3 ± 4.6	74.0 ± 2.6	76.4 ± 4.3
Intact acrosome	76.6 ± 2.8	77.7 ± 3.3	76.0 ± 3.6	75.9 ± 4.6	74.9 ± 3.7	73.9 ± 5.3

* Intact = viable, intact acrosome.

All measured sperm-quality parameters decreased during staining and incubation, regardless of whether incubation was performed at 15 °C or 37 °C (Wilcoxon signed rank test: TM, sperm integrity, sperm viability, acrosome integrity: P = 0.002 [15 °C/37 °C]). However, after staining with Hoechst 33342 and incubation at 15 °C for 4 to 6 h, all sperm-quality parameters were significantly higher than those after incubation of different samples at 37 °C for 1 to 1.5 h (Mann-Whitney U-test: TM, P = 0.023; sperm integrity, P = 0.001; sperm viability, P = 0.005; acrosome integrity, P = 0.0004). The initial quality parameters of ejaculates after collection did not differ between the two groups (Mann-Whitney U-test: P ≥ 0.238).

After incubation at 37 °C, high sample viscosity (n = 3) and the absence of a split into two populations in the flow cytometer (n = 4) hindered the sortability of the samples. Only three samples could be separated into X- and Y-chromosome–bearing sperm populations. After incubation at 15 °C, six samples were resolved into X- and Y-chromosome bearing sperm populations from which two could not be sorted due to low sample volume. High viscosity (n = 2) and the absence of resolution in two populations in the flow cytometer (n = 2) again prevented testing for sortability. The quality parameters of spermatozoa after staining and incubation at 37 °C and 15 °C, respectively, and sex sorting are presented in Table 2. Due to low number of

sorted samples (37 °C, n = 3; 15 °C, n = 4), no statistical testing was performed.

The event rate ranged from 1000 to 15,000 spermatozoa/sec and the sort rate was 300 to 700 spermatozoa/sec. The mean percentage of correctly oriented cells in sortable samples was 30.8 ± 13.8%. Determined sort purity was 94% and 75% for X- and Y-chromosome–bearing populations, respectively.

3.3. Experiment 3: Characterization of viscous seminal plasma fraction component

From 19 ejaculates collected from WR bulls, 12 were classified as viscosity grade II, five as viscosity grade III, and two were not viscous (grade I). Regarding the seven ejaculates collected from BR bulls, two were classified grade II and the other five showed no noticeable viscosity (grade I). Characterization of proteins from six rhinoceros seminal plasma samples of different viscosity (I to III) by SDS-PAGE and 2-DE, showed a highly acidic (Ip ≤ 3) 250-kDa protein spot (P250), whose occurrence and intensity strongly correlated with the grade of viscosity (Figs. 1 and 2). One-dimensional and two-dimensional electrophoresis gels displaying the protein composition of secretions from the bulbourethral glands, the prostate and the vesicular glands were searched for a 250-kDa protein band to determine the origin of P250. The only suitable
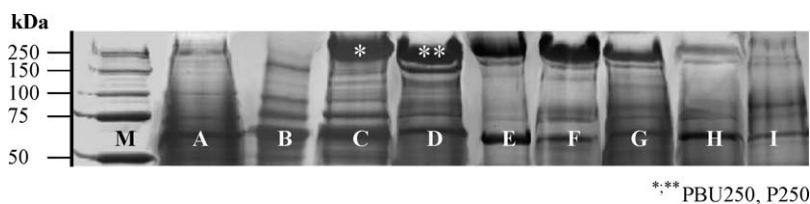
Fig. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern of accessory sex glands secretions and whole seminal plasma from ejaculates of different bulls and of different viscosity. Lane M: protein marker. Lane A: vesicular gland secretion. Lane B: prostate secretion. Lane C: bulbourethral gland secretion. Lanes D–F: seminal plasma viscosity grade III. Lane G: seminal plasma viscosity grade II. Lanes H and I: not viscous seminal plasma. *,**PBU250, P250.
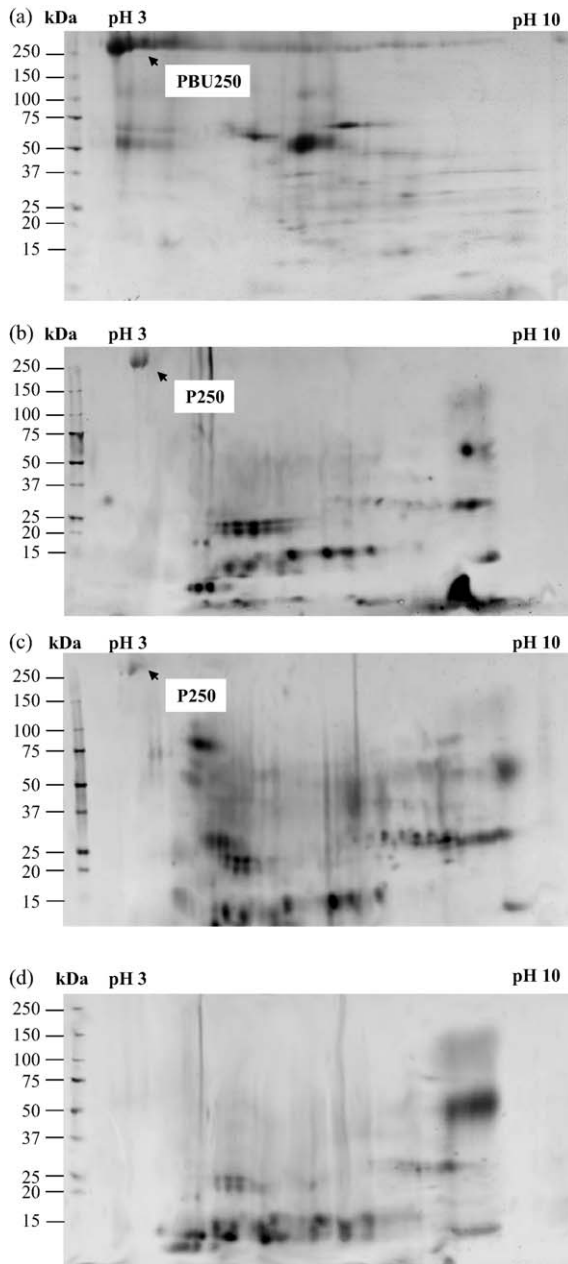
Fig. 2. Two-dimensional gel electrophoresis of (a) bulbourethral gland secretion displaying protein PBU250 as highly acidic protein spot, (b) viscous rhinoceros seminal plasma (III) displaying P250 as highly acidic protein spot, (c) viscous rhinoceros seminal plasma (II) displaying P250 as weak acidic protein spot, (d) not viscous rhinoceros seminal plasma (I) not displaying P250.

protein spot that was detected originated from the bulbourethral gland secretions (PBU250).

To test analogousness of P250 (black and white rhinoceros) and PBU250, and for further characterization, the protein spots were analyzed by MALDI-TOF MS followed by data searching using MASCOT.

Although good MS spectra were generated, no matching protein was found in the database. However, both spots are likely to represent the same proteins as 11 of 12 peptides (± 0.1 m/z) were seen in the spectra of both proteins.

3.4. Experiment 4: Liquefaction of viscous fraction using α-amylase and collagenase and evaluation of the enzyme-influence on sperm characteristics

3.4.1. SDS-PAGE

The concentration of P250 decreased in the SDS-PAGE gel when different concentrations of α-amylase or collagenase dissolved in 10 μL PBS were added to seminal plasma of viscosity grades II and III. The extent of protein decrease depended on the amount of enzyme added. Addition of 3 IU α-amylase and 18.5 IU collagenase caused a complete removal of the P250 band. Figure 3 shows a SDS-PAGE gel displaying seminal plasma samples with and without addition of α-amylase and collagenase, respectively.

3.4.2. Effect of α-amylase and collagenase on viscosity of seminal plasma

Viscosity was quantified by estimating a rotational correlation time (τ_c) from ESR spectra of untreated, α-amylase- and collagenase-treated seminal plasma, respectively (0.5 and 3 IU per 100 μL seminal plasma; Fig. 4). Figure 4 reveals that viscosity was significantly reduced upon addition of both enzymes compared with that of the controls (exact Friedman test, Wilcoxon and Wilcox test; df = 2, p $\leq$ 0.01, n = 7). The τ_c-times of
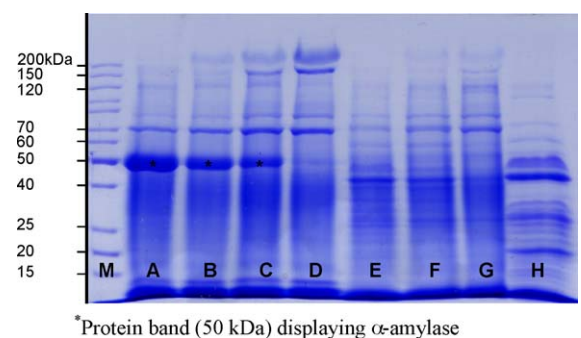
*Protein band (50 kDa) displaying α-amylase

Fig. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern of a viscous seminal plasma (III) sample from one white rhinoceros bull with and without addition of 3 concentrations of α-amylase or collagenase diluted in 10 μL PBS. Lane M: protein marker. Lanes A–C: seminal plasma with addition of 3 IU, 1.5 IU, and 0.5 IU α-amylase, respectively. Lane D: untreated seminal plasma. Lanes E–G: seminal plasma with addition of 18.5 IU, 9.25 IU, and 3.1 IU collagenase, respectively. Lane H: collagenase (40 IU). *Protein band (50 kDa) displaying α-amylase.
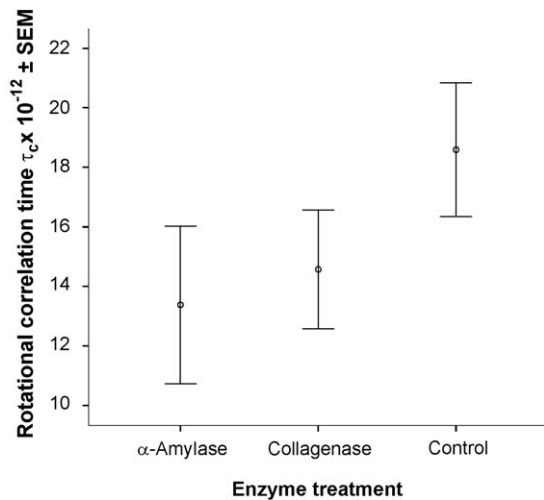
Fig. 4. Mean viscosity of α-amylase–treated (0.5 IU) or collagenase-treated (3 IU) and of control seminal plasma samples from white rhinoceros bulls, evaluated through determination of τ_c-times. Data are means ± SEM (n = 7).

control seminal plasma varied between 12.9×10^{-12} and 26.5×10^{-12} sec with a mean of 18.7×10^{-12} sec. After addition of α-amylase and collagenase, τ_c values were in the range of 5.81×10^{-12} to 14.70×10^{-12} sec (mean 13.2×10^{-12}) and 10.30×10^{-12} to 16.42×10^{-12} sec (mean 14.5×10^{-12}), respectively. Compared with untreated samples, addition of α-amylase or collagenase decreased viscosity of seminal plasma by 28% and 21%, respectively.

3.4.3. Impact of α-amylase and collagenase on quality parameters of spermatozoa

The influence of enzymes on PM, TM, and integrity (viability, acrosome integrity) was tested for α-amylase in 12 ejaculates (WR = 8, BR = 4) and for collagenase in eight ejaculates (WR = 7, BR = 1). The detailed results are presented in Table 3. No significant effect on sperm motility or integrity was found through addition of enzymes to semen samples compared with that of control samples after incubation for 1 h at RT (exact Friedman test: df = 4, $P \geq 0.187$).

4. Discussion

Sex-sorted spermatozoa suitable for AI or IVF might become an important management tool for captive wildlife populations as unbalanced sex ratios create difficulties with management of small populations, particularly in large, slow-reproducing mammals [50,51]. The methods developed in this study mark a

substantial step toward the successful application of sex-preselection technology in the rhinoceros.

The first challenge was to retain sperm function during transport (4 to 10 h) from collection site to sorting facility. Blottner's cryomedia [9,36] is routinely used for chilled storage of rhinoceros spermatozoa as motility is uniformly maintained over time periods similar to those used in this experiment [9]. Unfortunately, the high percentage of egg yolk in this medium (16%) interferes with DNA staining and resolution of spermatozoa during the sorting procedure, making this extender not suitable for sex sorting. Unlike chilling of rhinoceros semen diluted in Blottner's cryomedia, which maintains almost constant motility of spermatozoa, the decline in PM and TM observed in Experiment 1 for all non–egg yolk extenders indicates the importance of egg yolk in the protection of rhinoceros spermatozoa and maintenance of their motility during low-temperature liquid storage. In any case, of the egg yolk–free media tested, MES was best to maintain the motility of spermatozoa after transport and was classified suitable for sperm sorting in the rhinoceros, as reasonable resolution of X- and Y-chromosome–bearing sperm populations was achieved using this semen extender.

Staining during transport to the laboratory at 15 °C resulted in better quality of spermatozoa than staining at 37 °C after transport at 4 °C. Despite the sensitivity to chilling of rhinoceros spermatozoa, this finding might be due to an aversion to temperature changes or to exposure to high temperatures further complicating sperm sex-sorting in this species.

As wildlife have not been selected for high fertility and sperm production, sorting efficiencies in most exotic species are likely to remain below those observed in livestock species. Considering the sperm-sorting index of the rhinoceros, general sortability sits in the range between the human and the stallion, consistent with the medium degree of resolution of X- and Y-chromosome-bearing sperm populations [13]. However, sorting rates in the rhinoceros were low in the current study, with a maximum of 700 cells/sec compared with a mean of 3500 to 4500 cells/sec in the stallion [40], and only 30% (incubation at 37 °C) and 60% (incubation at 15 °C) of stained sperm samples were separable into X- and Y-chromosome-bearing sperm populations. These findings may be due to the great variance in semen quality between ejaculates resulting in a relatively low mean percentage of viable and intact spermatozoa after DNA staining and the well-established differences in sortability of spermatozoa between individual males [52]. Additionally, the low concentration of spermato-

zoa in some ejaculates complicated the staining procedure and reduced the mean event and sort rates.

High viscosity of semen samples interfered with the requirement of stable single-cell suspensions and even prevented the handling of 25% of stained sperm samples in the flow cytometer. A glycoprotein of 250 kDa molecular weight and an Ip of ≤3 was discovered, which is suggested to be the major component of the highly viscous fraction in the seminal plasma of the African rhinoceros. The results of peptide mass fingerprint analysis provided strong evidence that P250 originates from the bulbourethral gland secretions. The main functions of the secretion from these accessory glands are lubrication and neutralization of the urethra prior to ejaculation [21,53]. However, in the boar, glycoproteins derived from the bulbourethral glands form a gelatinous plug in the uterine cervix of the sow, which is believed to prevent backflow of the semen [54]. In camelids, the viscous seminal plasma entraps the spermatozoa in the uterus after ejaculation, possibly steadily releasing spermatozoa to allow fertilization for an extended period after the induction of ovulation [55]. In the rhinoceros, it is difficult to specify which task the bulbourethral gland secretion performs. To date, the only way to collect full ejaculates has been by electroejaculation. Therefore, it is possible that the highly viscous component is not a natural part of the ejaculate but rather an artifact of the artificial method of semen collection.

Bulbourethral glands are generally known to secrete mucin-like glycoproteins [53]. The dissolving effect of α-amylase suggests that P250 may be a glycoprotein, as the glycoside hydrolase α-amylase breaks down long-chain carbohydrates. However, P250 could not be identified, and no matching protein was found in the database, although good MS spectra were generated. Therefore, further investigation of protein structure is necessary.

The viscosity of analyzed semen samples was higher for the WR than for the BR. The reasons for this are not yet fully understood as anatomic features of reproductive tracts from these two closely related species are similar. Nevertheless, the bulbourethral glands of the WR are proportionally bigger in size and more elongated compared with that of the BR [22]. To find a way of decreasing the viscosity of semen without damaging the spermatozoa, the impact of the enzymes α-amylase and collagenase was investigated. By employing an ESR approach, a significant decrease of a rotational correlation time of the spin label Tempo was observed in seminal plasma treated with α-amylase or collagenase in comparison with control plasma.

Although a detailed calculation of the solution viscosity from τ_c values depends on several molecular parameters (size of the label compared with that of surrounding solvent molecules, interaction between label and solvent molecules [46]), these data clearly show that upon addition of the enzymes, the viscosity of seminal plasma was decreased. No negative impact of enzymes on sperm-quality parameters (sperm motility, viability, acrosome integrity) was observed. Nevertheless, prior to a routine addition of the enzymes to viscous rhinoceros ejaculates, their impact on function and fertility of spermatozoa has to be further investigated. Both enzymes completely dissolved P250 when added at the highest concentration tested (Fig. 3). Thereby, despite P250, also further high-molecular proteins were degraded (≥70 kDa; Fig. 3) rendering an impact of these proteins on seminal plasma viscosity possible next to the dominant P250. To verify a potential involvement of these proteins for instance in the form of an interaction of several seminal plasma components as described in the boar [56] and the rat [57], further investigations on the fine structure of rhinoceros seminal plasma are required.

In the current study, rhinoceros spermatozoa were sex-sorted for the first time into high-purity X- and Y-chromosome-bearing sperm populations. Nevertheless, sample quality and sorting efficiency prevented the use of the sex-sorted samples for deep intrauterine AI. However, adequate numbers of spermatozoa could be easily sex-sorted for ICSI or IVF. Recently, in vivo collection of an average of six oocytes per procedure was achieved in the black and the white rhinoceros using ultrasound-guided transvaginal laparoscopic follicular aspiration [58].

Furthermore, a glycoprotein of 250 kDa molecular weight, most likely originating from the bulbourethral glands, was identified and correlated with the viscosity of ejaculates. Its liquefaction was achieved using the enzymes α-amylase and collagenase without any effect on the quality parameters of spermatozoa. Such enzyme treatment may allow better use of rhinoceros ejaculates for sex sorting by flow cytometry, but further studies are required to confirm this.

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