Available online at www.sciencedirect.com
ScienceDirect
Theriogenology 71 (2009) 275–291
Theriogenology
www.theriojournal.com

Split-sample comparison of directional and liquid nitrogen vapour freezing method on post-thaw semen quality in white rhinoceroses (*Ceratotherium simum simum* and *Ceratotherium simum cottoni*)

C.E. Reid [a,1,*], R. Hermes [a,1], S. Blottner [a], F. Goeritz [a], G. Wibbelt [a], C. Walzer [b], B.R. Bryant [c], T.J. Portas [c], W.J. Streich [a], T.B. Hildebrandt [a]

[a] *Institute for Zoo and Wildlife Research, Alfred-Kowalke-Strasse 17, 10315 Berlin, Germany*
[b] *Institute of Animal Ecology, University of Veterinary Medicine, A-1160 Vienna, Austria*
[c] *Western Plains Zoo, P.O. Box 831, Obley Road, Dubbo, NSW 2830, Australia*

Received 26 March 2008; received in revised form 7 July 2008; accepted 12 July 2008

Abstract

To increase the quality of cryopreserved sperm in white rhinoceros, the liquid nitrogen vapour (LN vapour) freezing and the multi-thermal gradient directional freezing methods were compared. Sixteen white rhinoceros (*Ceratotherium simum* sp.) were electro-ejaculated. Semen samples were diluted with cryoextender (Tris, lactose, egg-yolk, DMSO) and aliquoted into straws for LN vapour freezing, and glass hollow tubes for directional freezing. The sperm quality was evaluated before and after freezing by assessing the following parameters: motility, morphologic state, acrosomal integrity and plasma membrane function and integrity (i.e. sperm viability) as defined by the hypo-osmotic swelling. Directional freezing improved the sperm viability by 5.6% ($p < 0.005$), progressive motility score by 34.7% and sperm motility index (SMI) by 8.1% ($p < 0.005$) versus LN vapour freezing. When data was categorized into groups of low (<19%), moderate (20–39%) and high (>40%) percentages of morphologically normal, directional freezing (DF) resulted in 31.4% less abnormal acrosomes for the low quality group as well as 18.7% increase in intact acrosomes and 10.9% increase in motility for the high quality group compared to LN vapour freezing (LN) ($p < 0.01, p < 0.03, p < 0.01$, respectively). LN showed a significant reduction in sperm head volume (5.7%, $p < 0.05$) compared to the prefreeze; whereas, no significant reduction in head volume was demonstrated after DF. Several additives (xanthenuric acid, cytochalasin D, potassium, EDTA) to the basic cryoextender provided no significant improvement in spermatozoal survival after directional freezing. In conclusion, directional freezing proved to facilitate higher gamete survival compared to LN vapour freezing. This is especially effective in ejaculates of low sperm quality and is important in endangered species where high quality semen donors are often not accessible. These results suggest that directional freezing could be valuable particularly for species with limited freezability of spermatozoa.

Keywords: Reproduction; Rhinoceros; Sperm cryopreservation; Directional freezing

1. Introduction

Declining natural habitats, human–animal conflicts and illegal poaching of rhinoceros species and subspecies have already lead to the extinction of several subspecies (Sumatran rhinoceros subspecies: *Dicerorhinus sumatrensis lasiotis*, Javan rhinoceros subspecies:

* Corresponding author. Tel.: +49 30 516 8000; fax: +49 30 512 6104.
E-mail address: reid@izw-berlin.de (C.E. Reid).
[1] Authors have made equal scientific contributions to this study and therefore share first-authorship on this manuscript.

0093-691X/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2008.07.009

Rhinoceros sondaicus inermis (www.rhino-irf.org, September 2006), ''western'' black rhinoceros subspecies: *Diceros bicornis longipes* (www.iucn.org press release September 2006)). The northern white rhinoceros (*Ceratotherium simum cottoni*) is reported to have only 4 animals remaining in the wild population, and therefore, is considered demographically extinct (www.iucn.org press release September 2006). Because of the situation in the wild, self-sustaining captive populations of endangered rhinoceros species are of extreme importance for the survival of the species. It is highly likely that in the next decades some of the rhinoceros species and sub-species will only exist in captivity. Therefore if efforts are not immediately taken to establish self-sustaining captive populations, these rhinoceros species and subspecies will only exist as skeletons in the natural history museums of the world.

The implementation of assisted reproduction techniques (ART) and gamete preservation are imperative for establishing self-sustaining captive populations while maintaining genetic diversity. On a practical basis, the use of ART prevents the high risk transport of animals for mating encounters and expands the ability to increase the genetic potential of under represented individuals. ART using fresh semen has already been developed and has proven successful in southern white rhinoceroses [1]. The use of cryopreserved spermatozoa with optimal post-thaw quality is paramount for further implementation of assisted reproduction.

The use of cryopreserved sperm would overcome current logistical problems of using fresh semen for AI and long distances between donor and receiving female. Additionally, it would permit the use of genes from wild populations without animal importation as well as the use of genetic material after the death of non-represented animals.

Spermatologic information available for rhinoceros species is limited. Spermatological parameters have been described for all 4 rhinoceros species in captivity, but cryopreservation has as of yet only been described for the 3 rhinoceros species, black, white and Indian [2] with a range of different freezing and collection methods (Black, ejaculated: [3]; white, epididymal: 4,5; Sumatran and Black, post-coital and epididymal: 6; white, ejaculated: 7). The results vary between 1 and 6% [4,5] and 50% [5] post-thaw total motility. A sperm motility index (SMI) of 47.3 was reported for post-coitally collected Sumatran rhino semen and 41.3 and 47.3 SMI for black rhino epididymal sperm cryopreserved with glycerol and dimethyl sulfoxide (DMSO), respectively [6], but is not comparable to total motility. Throughout these studies various cryopreservation techniques, cyropreservants, and assessment techniques were used making it impossible to compare the results. In addition, with the exception of Hermes et al. [7] using 21 animals, these studies were based on 1 or 2 individuals or even only 1–2 samples and are therefore, very difficult to interpret.

Cryopreservation in the rhino is described using LN vapour freezing methods (LN): a dry shipper and the dry ice [6] and liquid nitrogen vapour techniques [7,4,5]. These methods, although antiquated are the current standard for zoo and wildlife reproduction probably because of the ease and simplicity of the protocols and the minimal cost of investment for equipment. All three methods are based on using multi-directional (equiaxed) heat transfer to achieve a rate of temperature change in the sample that is dependent on the thermal conductivity and geometrical shape of the container and of the biological material within it. The thermal gradient within the sample is determined implicitly by the temperature of the chamber and the thermal conductivity of the materials of the sample, and is not directly controllable. Thus, ice grows at an uncontrolled velocity and morphology disrupting and killing many cells in the sample [8].

There are many newer computer-based technologies to have been shown to improve post-thaw quality; however, these technologies typically have large, sensitive machinery that is not conducive to transport and use outside a lab environment. In addition, they also have significantly higher learning curves are relatively more expensive and require continued maintenance. The directional freezing technology (DF), although also seemingly expensive, is more durable for movement and use outside the laboratory, is easy to use and requires little to no maintenance. This technology (DF) is based on a ''multi-thermal-gradient'' directional solidification, in which the cryo-tube is moved at a constant velocity through a linear temperature gradient; thus, the cooling rate and ice front propagation and morphology are precisely controlled. The production animal industry has demonstrated a high rate of success using DF [8–12]. To date, three studies have been published comparing the new DF technology and the LN vapour freezing technique. Eight reports increased post-thaw motility and a comparable number of successful pregnancies in cows after a double freezing/thawing cycle using DF and single freezing/thawing cycle using LN method. Zirkler et al. [12] reported no differences in total or progressive motility, functional plasma membrane integrity, acrosome integrity, mitochondrial status and DNA fragmentation in stallions before and after both freezing methods. O'Brien and Robeck [13], found enhanced *in vitro* quality of sorted and non-sorted spermatozoa of bottlenose dolphins with DF compared to LN.

The objective of this study is to comprehensively compare the LN and DF technology and evaluate the potential benefits for its use in the cryopreservation of white rhinoceros sperm, as well as justify the significant expense. Further, semen extender additives shown to improve the post-thaw quality of stallion spermatozoa were tested and comprehensively evaluated for both freezing methods.

2. Methods and materials

2.1. Animals

Thirteen sexually mature (>7 years old) southern white rhinoceros (*Ceratotherium simum simum*) bulls, three male northern white rhinoceros (*C.s. cottoni*) housed in zoological institution in Europe, Australia and the US were selected for this study (Table 1). All animals were listed in the European and North American Species Survival Programs (SSP). The examined bulls were randomly selected and included non-proven ($n = 12$) and proven breeders ($n = 4$) as well as wild caught to third generation captive born individuals (F0 $n = 10$, F1 $n = 3$, F2 $n = 2$, F3 $n = 1$). Because the repeatability of semen characteristics between ejaculates has been reported to be low to moderate [14,15], multiple ejaculates (2–5 ejaculates/per bull) from all but 5 bulls were obtained to increase the accuracy of the semen quality estimates. Multiple ejaculations of five bulls were not possible due to uncontrollable logistic reasons. The results for multiple ejaculates from the same individual were first averaged before being included in the complete data base. All substances were supplied by Sigma–Aldrich unless otherwise indicated (Steinheim, Germany).

2.2. Split-sample comparison of directional and LN vapour freezing

Semen was collected using electro-ejaculation [7]. A 16% egg yolk, lactose based, 6% DMSO extender was used as control medium for the comparison of DF and LN. This extender has been successfully used to cryopreserve semen of several wild animal species and was further specifically described for rhinoceros [16,7].

Control medium, all equipment and semen were maintained at 37 °C using an incubator or water bath until semen was extended. The color, pH and odor were evaluated immediately after the collection of each fraction. Semen was immediately extended 1:1 with washing medium for the centrifugation, which was prepared by omitting alpha-tocopherol and DMSO from control medium preparat.

The progressive and total motility of extended native semen was evaluated. Fractions with similar total and progressive motilities were combined, fractions with substantially different progressive and total motilities were processed separately and fractions <50% motility were discarded. Aliquots for the evaluation of concentration, membrane integrity, normal morphology and acrosome integrity were taken from each probe, which was then, cushioned with 1 ml Optiprep™ and centrifuged at speeds of $1000 \times g$ for 10 min to remove seminal plasma. After centrifugation, the supernatant with seminal plasma was discarded. The resulting spermatozoal band was aspirated and re-extended to a concentration of approximately 68×10^6 sperm/ml with control medium. The resulting probe was split into two equal portions for directional freezing (DF) and LN vapour freezing (LN). Probes were placed in 2 ml and 10 ml glass hollow tubes and 0.5 ml straws for DF and LN, respectively. The prepared probes were then placed in a room temperature water bath and equilibrated for 2 h to 4 °C at a rate of −0.3 °C/min using a passive cooling device prior to freezing.

2.3. Directional freezing

Probes were packaged in 2 ml and 10 ml glass tubes (HollowTube™, IMT Ltd., Ness Ziona, Israel) 4 and 10 cm long, respectively, with a 1.5 cm total diameter and a hollow centre (0.5 cm diameter) and sealed with silicon rubber stoppers (Harmony CryoCare™, IMT Ltd., Ness Ziona, Israel). Tubes were frozen using the MTG 516 (Harmony CryoCare™, IMT Ltd., Ness Ziona, Israel). The start temperature was 4 °C, the end temperature was −50 °C and the velocity was 1.0 mm/s: therefore the linear temperature gradient was 5.5 °C/mm and the subsequent freezing rate was −33 °C/min. Seeding was done automatically for 100 s at −50 °C. After DF was complete, the tubes were plunged and stored in liquid nitrogen for at least 48 h before thawing. Directional freezing is possible using straws as well as other volumes and settings; however, preliminary trail results indicated that the highest post-thaw semen quality resulted with the use of glass tubes and the volumes and setting stated above. Preliminary trail results are not included in this manuscript.

To thaw, tubes were passively warmed at room temperature for 60 s, followed by active warming for 30 s in a 37 °C water bath (Harmony CryoCare™, IMT Ltd., Ness Ziona, Israel) to achieve a final temperature that was approximately room temperature. Thawed-

Table 1
Initial semen assessment of examined rhinoceros bulls

Species	Animal ID	Ejaculated	Volume (ml)	Concentration (10^6)	pH	Total motility (%)	Progressive motility	SMI
C.s.s	LOK	1	54	440	–	62.6		
		2	32	640	–	85	3	72.5
		3			–			
	MBB	1	7.3	200	10	70	2	40
	DSA	1	39.5	50	7.8	80	4	80
		2	100	190	7.8	67	4	73.5
	EBH	1	5	65	7.5	90	4	84.5
		2	15	175	7.5	84	4	82
		3	30	25	7.5	84	4	84
	HMD[a]	1	0.3	100	8.1	90	4	85
		2	0.3	375	7.8	74	4	77
	KDA	1	13.5	80	–	88	5	94
	SCUK	1	56	10	7.5	90	5	95
		2	27.5	80	7.2	90	3	75
		3	28.25	80		90	3	75
	UDA	1	2	80	7.2	72	5	86
	WLUK	1	13	85	7.5	72	3	66
		2	15	35	7.5	69.5	3	64.75
	SWMUK	1	57	454	7.5	90	3	75
		2	22	454	7.5	93	3	76.5
	WLPF	1	20	3	8	90	4	85
		2	10	1	8	95	4	87.5
		3	22	1	8	95	4	87.5
	DUCR	1	45.5	15800[b]	9.5	90	4	55
	GBB	1	1.6	106	8	74	2	57
C.s.c	ASD	1	3.5	150	7.3	82	5	91.5
		2	15	11	7.5	63	2	91.55
		3		10	7.5	89	1	54.5
	SIIDCR	1	0.4	500	10	85	4	52.5
		2	44	500	10	90	4	52.5
	SDCR	1	35.1	200	10	70	2	40
		2	7	115		65	4	72.5
		3	75	30		85	5	92.5
		4	17	20		78	5	93.5
		5	16					
	Range		0.4–100	1–640	7.2–10	50–95	1–5	40–95
	Mean		29.86	164.53	9	82.58	3.63	72.56
	S.E.M.		4.50	31.83	0.2	1.63	1.03	2.96

Studbook numbers and animal names are not revealed to preserve privacy.
[a] Bull HMD was manually stimulated, whereas the other bulls were electro-ejaculated; and therefore not included in the calculation of the average volume.
[b] DUCR concentration was abnormally high and determined to be an outlier, according to the one sample *t*-test; and therefore, not included in the calculation of the average concentration.

semen was re-extended 1:0.5 (thawed semen:extender for an end volume of 1.5 ml) with washing medium plus 0.4% poly-vinyl-pyrolidone (PVP, end concentration of 1.3% PVP) and evaluated after 5–15 min equilibration at 37 °C.

2.4. LN vapour freezing

Although volumes equivalent to that used in DF are available for the LN vapour freezing method, preliminary trail results indicated that 0.5 ml straw

Table 2
Native samples categorized according to percentage of normosperm/ejaculate

	Normal morphology (%)	Double defects (%)	Intact acrosomes (%)	HOS	TZI	SDI
Low quality (NMorph <19%)	11.66 ± 2.3^{a}	32.66 ± 9.81^{b}	$23 \pm 4.49^{c,d}$	$19.5 \pm 4.91^{f,g}$	1.40 ± 0.10	1.21 ± 0.30
Moderate quality (NMorph 20–39%)	26.42 ± 2.71^{a}	19.57 ± 4.04^{b}	$40.16 \pm 5.89^{c,e}$	48.83 ± 12.4^{f}	1.27 ± 0.05	0.93 ± 0.04
High quality (NMorph >40%)	53.41 ± 1.68^{a}	12.47 ± 1.57^{b}	$58.4 \pm 2.23^{c,d,e}$	58.76 ± 3.16^{g}	1.27 ± 0.03	0.62 ± 0.03

Normospermic parameters for native/pre-freeze semen categorized according to percent normal morphology. Values are reported as absolute values + standard error of the mean. Values with like significant differences are indicated by common letters. Values in each column with the same letters have common significant differences. $^{a,d,g}p < 0.001$. $^{c}p < 0.01$. $^{b,e,f}p < 0.05$.

volumes resulted in the highest post-thaw semen quality in white rhinoceroses. Preliminary trail results also indicated that the highest post-thaw semen quality was obtained at a height of 2 cm above the LN vapour. Results from preliminary trails are not included.

Probe samples were frozen in 0.5 ml straws (Minitube GmbH, 84184 Tiefenbach, Germany) 2 cm over −80 °C liquid nitrogen vapour for 10 min before being plunged into liquid nitrogen. The resulting freezing rate was −8 °C/min. Straws were stored in liquid nitrogen for at least 48 h before thawing.

Semen straws were thawed in a 38 °C water bath for 60 s, re-extended 1:0.5 (thawed semen:extender for an end volume of 1.5 ml) with washing medium plus 0.4% PVP (end concentration of 1.3% PVP) and evaluated after 5–15 min equilibration at 37 °C.

2.5. Effects of directional and LN vapour freezing on different pre-freeze sperm quality groups

For conductive comparison of the effects of DF and LN on different states of pre-freeze spermatozoal quality, samples were categorized into three quality status groups based on percent of normal morphology [17]: low (<19%), moderate (20–39%), and high (>40%) and thus as a consequence also differing in, percent intact acrosomes, percent double defects, and percent viable sperm (Table 2). Statistical comparison of the means for sperm quality parameters among the three groups before and after each freezing method were used to detect any positive effects of DF and/or LN among varying spermatozoal quality. Averages of raw data are reported as log 10 (Table 3).

Table 3
Post-thaw results of split-samples comparision categorized according to percentage of normosperm/ejaculate

	Low quality			Moderate quality			High quality		
	Directional freezing	Conventional freezing	Significance	Directional freezing	Conventional freezing	Significance	Directional freezing	Conventional freezing	Significance
Normal morphology	0.74 ± 0.18	0.61 ± 0.31	NS	1.39 ± 0.05	1.04 ± 0.20	NS	1.37 ± 0.06	1.25 ± 0.11	NS
Intact acrosomes	0.59 ± 0.18	0.45 ± 0.15	NS	1.50 ± 0.03	1.23 ± 0.21	NS	1.5 ± 0.05	1.22 ± 0.10	$p < 0.05$
No acrosomes	1.04 ± 0.14	1.42 ± 0.15	NS	0.68 ± 0.15	0.88 ± 0.28	NS	0.92 ± 0.17	1.05 ± 0.09	NS
Abnormal acrosomes	1.10 ± 0.15	1.61 ± 0.08	$p < 0.01$	1.64 ± 0.05	1.47 ± 0.21	NS	1.56 ± 0.02	1.52 ± 0.05	NS
Tail defects	1.29 ± 0.24	1.18 ± 0.31	NS	1.16 ± 0.24	1.18 ± 0.19	NS	0.98 ± 0.11	1.17 ± 0.07	NS
No tails	0.83 ± 0.17	1.17 ± 0.28	NS	0.44 ± 0.11	0.78 ± 0.45	NS	0.81 ± 0.10	0.92 ± 0.11	NS
Double defects	1.36 ± 0.18	1.78 ± 0.21	NS	1.34 ± 0.08	1.44 ± 0.13	NS	1.22 ± 0.08	1.26 ± 0.08	NS
HOS	1.35 ± 0.27	1.13 ± 0.57	NS	1.39 ± 0.07	1.78 ± 0.13	NS	1.64 ± 0.05	1.72 ± 0.05	NS
Total motility	1.37 ± 0.12	1.13 ± 0.10	NS	1.74 ± 0.06	1.42 ± 0.14	NS	1.69 ± 0.03	1.53 ± 0.15	NS
Progessive motility	2.8 ± 0.47	1.50 ± 0.50	NS	4 ± 0.40	2.75 ± 0.85	NS	3.5 ± 0.26	2.63 ± 0.27	$p < 0.05$
SMI	1.56 ± 0.09	1.32 ± 0.20	NS	1.84 ± 0.05	1.62 ± 0.18	NS	1.69 ± 0.05	1.50 ± 0.04	$p < 0.05$
TZI	1.34 ± 0.10	1.80 ± 0.29	NS	1.29 ± 0.04	1.36 ± 0.07	NS	1.26 ± 0.03	1.27 ± 0.03	NS
SDI	1.24 ± 0.13	1.6 ± 0.11	NS	1.04 ± 0.1	1.17 ± 0.12	NS	0.92 ± 0.06	0.99 ± 0.09	NS

Values are reported as log base 10 of the mean + standard error of the mean.

2.6. Extender additives

Five cryomedia variations were formulated using control medium as a base. Variant 1 (KEDTA) was prepared by adding 10 mmol KCl and 3.0 mmol EDTA and variant 3 (CYTOD) was prepared by adding 9.8×10^{-3} mmol cytochalsin D. Variant 2 (XAN) was prepared by substituting 5.0 mmol xanthurenic acid for alpha-tocopherol. Variant 4 (ALL) was prepared by substituting 5.0 mmol xanthurenic acid for alpha-tocopherol and adding 10 mmol KCl, 3.0 mmol EDTA, and 9.8×10^{-3} mmol cytochalsin D. Variant 5 (2DMSO) was prepared by omitting DMSO. The latter mixture was then divided into two equal parts. The first half of this mixture served as Step-1 of the dilution process (2aDMSO). To the other half of this mixture, 12.5% (v/v) DMSO was added and the resulting mixture served as Step-2 of the dilution process (2bDMSO). All extenders were prepared and stored at −80° C until used. Extenders were thawed in a 37 °C water bath and sonificated (ELMA T220, Singen, Germany) for 30 min shortly before use [18,19].

Aliquots for the evaluation of concentration, membrane integrity, normal morphology and acrosome integrity were taken from each fresh semen probe, which were separated into 6 equal portions and processed as described for split-sample comparison except that after centrifugation only five of the six resulting spermatozoa bands were re-extended with control medium, KEDTA, CYTOD, XAN, and ALL (Fig. 1). The resulting probes were further divided into two equal portions; one for DF and one for LN. The sixth sperm band were re-suspended with the DMSO-free 2aDMSO to a concentration of 136×10^6 sperma-spermatozoa/ml and equilibrated as described above. Prior to freezing, the re-suspended sixth pellet was further diluted with the pre-cooled (4 °C) 12.5% DMSO containing 2bDMSO to a final concentration of 68×10^6 spermatozoa/ml and with 6% DMSO (v/v). The probe was then divided into two equal parts, placed in 2 ml glass hollow tubes and 0.5 ml straws and immediately frozen using DF and LN, respectively (Fig. 1). Extenders were compared separately for each freezing method.

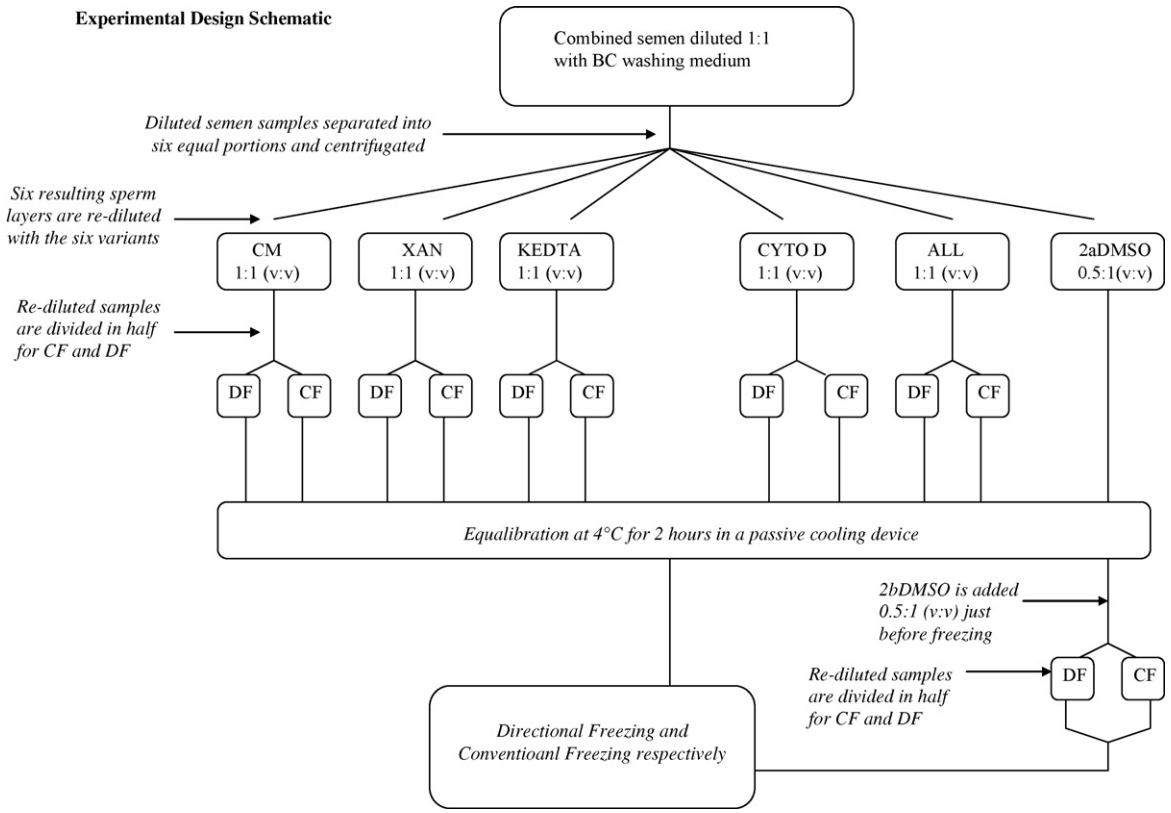

Fig. 1. Schematic of the experiment design for the extender study the comparison of directional and LN vapour freezing. Note that variant 2aDMSO is the only extender added with a two-step dilution method.

2.7. Calculation of putative fertility

Curves of putative fertility were adapted from the stallion because of the close evolutionary relationship to the rhinoceros and the wide range of fertility information available. The fertility study by the Pesch et al. [17] was specifically chosen because of its broad study group (i.e. included fertile, subfertile and infertile individuals), which best reflected the captive rhinoceros population in the present study. Using the Spearman rank correlation coefficient ($r_s = -0.562$, $p < 0.001$) and the regression line ($y = -0.904x + 100.1$) for fertility against pathomorphology in stallions [17], the predicted pre-freeze and post-thaw fertility was calculated for both freezing methods in the rhinoceros.

2.8. Spermatozoal assessment

Most sperm assessment methods were selected based on ease of procedure and adaptability of respective protocols for use in the field as well as reduced expense. The reasoning for this was to ensure that protocols could be easily replicated and adapted by zoo organizations that may not have access to standard CASA or flow-cytometry type assessment tools. All methods have been proven and shown to be accurate, reliable and reproducible. Assessment protocols were standardized in accordance with the World Health Organization (WHO) *Laboratory Manual* (4th edition, 1999). Basic macroscopic and microscopic examinations included concentration, motility, morphology and volume reaction in hypotonic medium.

2.9. Motility

The motility was evaluated (phase-contrast microscopy) as percentage of total motility and progressive motility based on a 5 point scale: 1 = l stationary movement, 5 = rapid forward movement, 2–4 are intermediate forms of propulsivity. A pre- and post-thaw sperm motility index was calculated using the following equation according to the WHO guidelines (4th edition, 1999) [20].

$$\mathrm{SMI} = \frac{(\text{percentage total motility} + (\text{forward progressive motility} \times 20))}{2}.$$

2.10. Morphology

Slides for sperm morphology evaluation were also prepared in accordance with the WHO [20]. The morphologic integrity of 200 spermatozoa was evaluated after staining with Congo red tannic acid and brilliant cresyl blue as previously described for cattle and rhinoceros ([16,7], respectively) Spermatozoa were categorized as intact or abnormal cells. Abnormalities included defects of the head and midpiece/tail: I = normal, II = abnormal acrosome, III = reacted acrosome, IV = no tail, V = tail/midpiece defect, VI = multiple abnormalities, in which each specific abnormality was recorded (Fig. 2). Teratozoospermic index (TZI) and the sperm deformity index (SDI) were calculated in accordance with WHO (4th edition, 1999) [20].

$$\mathrm{TZI} = \frac{\text{total number of defects}}{\text{total number of sperm with defects}}$$

$$\mathrm{SDI} = \frac{\text{total number of defects}}{\text{total number of sperm counted}}$$

2.11. Acrosome integrity

Acrosome integrity was evaluated using a fluorescence lectin dye method (i.e. FITC conjugated peanut agglutinin) specific to stain the acrosome. Staining technique is a modification from Blottner et al. [21] and O'Brien and Roth [6]. Staining was done after alcohol-fixation (30 min in 90% ethanol) of pre-prepared air-dried smears. After drying of the fixed smears, a 10 μl droplet of FITC-PNA (5×10^{-4} mg/μl) was gently spread over the smear and incubated in the dark at 4 °C for up to 6 h. The slide was then quickly rinsed with 1 ml of PBS and a coverslide was placed on the wet slide. The acrosome integrity was then evaluated using a fluorescence microscope with filters for excitation at wavelength 480 and emission at wavelength 535. Acrosomes were classified as I = intact, II and III are intermediate forms, IV = reacted (Fig. 3).

2.12. Viability/plasma membrane integrity and functionality

The cell viability was determined based on the plasma membrane function and integrity, which was evaluated using the HOS test. The method was adapted from Van der Ven et al. [22] with some modifications using the diluted lactose-buffer base of control medium. Twenty microliters of native semen were diluted in 100 μl with an osmolarity of 100 mOsm and incubated for 30 min at 37 °C. Subsequently, the spermatozoa were fixed with para-formaldehyde (18.5%, 25 μl) and stored at 4–6 °C until analyzed. From each sample two slides were prepared with 15 μl and 100 sperm per slide were

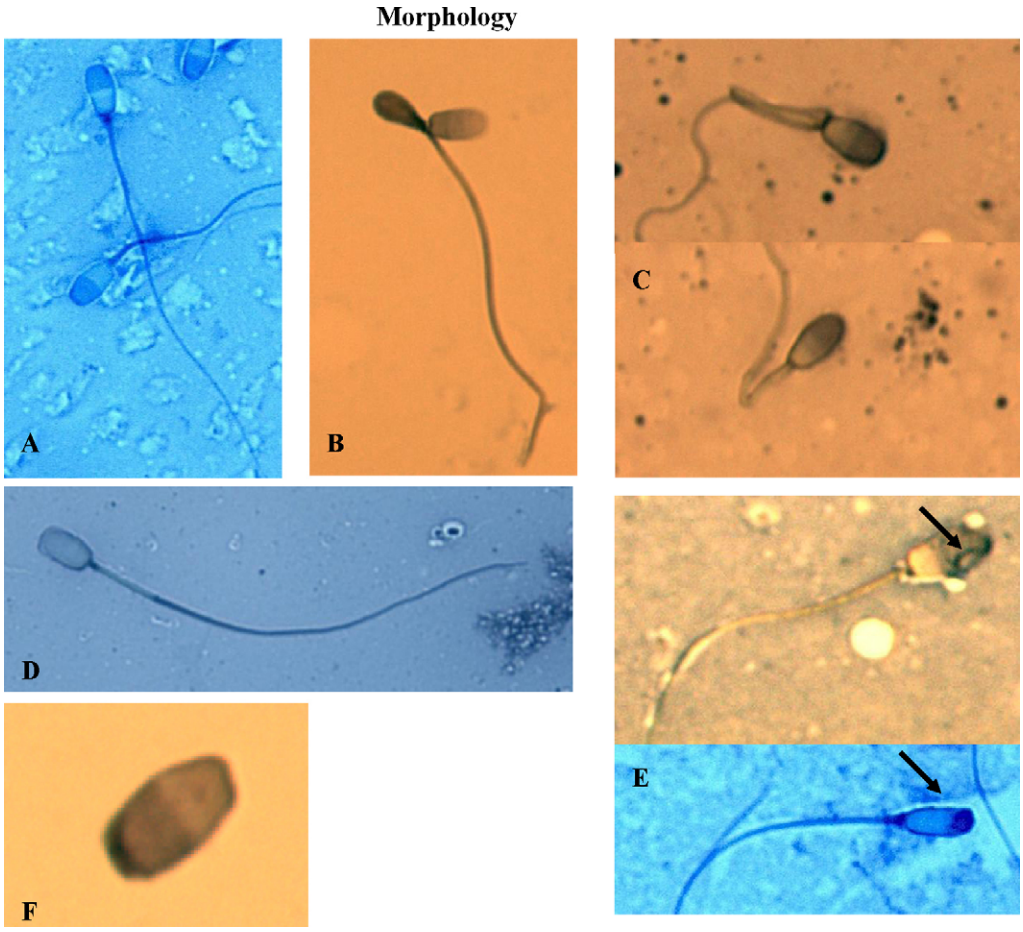

Fig. 2. Pictures illustrating the 6 morphological abnormalities evaluated in this study. (A) Normal spermatozoa with normal acrosome, tail and mid-piece, (B) spermatozoa with double defects, i.e. tail defect and reacted acrosome, (C) two spermatozoa with tail/mid-piece defects, the top picture indicates a tail defect and the bottom picture indicates a mid-piece defect, (D) a spermatozoa with reacted acrosomes, (E) two spermatozoa with abnormal acrosomes, the lower picture shows a vacuolated acrosomes, the top picture shows a partly dissolved acrosome and (F) a spermatozoa head with normal acrosome and no tail.

evaluated. The dead spermatozoa or unchanged cells were categorized as HOS negative and swollen cells or cells with curved swollen tails thus viable cells with functional membranes were HOS positive (Fig. 4). HOS was corrected for percent of morphological tail defects measured in the corresponding untreated sample.

2.13. Effects of directional and LN vapour freezing on cell expansion

To evaluate the potential effects of the two freezing methods on cellular expansion as a result of varying osmotic pressures through the cryopresevation process, head volume from 100 air-dried, trichrom-stained, morphologically normal spermatozoa was measured in the high quality semen group with >40% normal morphology using an Axioplan Zeiss microscope with a Plan Neofluar 100×/1.30 Oil immersion lens and mounted Dig 3300 camera (Jena, Germany). Still pictures of 100 normal spermatozoa from each sample were taken and head volume was measured using the CELL Soft Imaging System Software (Olympus, Hamburg, Germany). The average of the head volume averages per sample before and after both freezing methods where statistically compared.

2.14. Statistical analysis

All statistics were performed using (GraphPad InStat Version 3.00, GraphPad Software, San Diego, CA, USA, www.graphpad.com). When statistical distribution of the data group was not normal (i.e. skewed right/left) and/or significant differences existed among the standard deviations (S.D.) of the means, data was

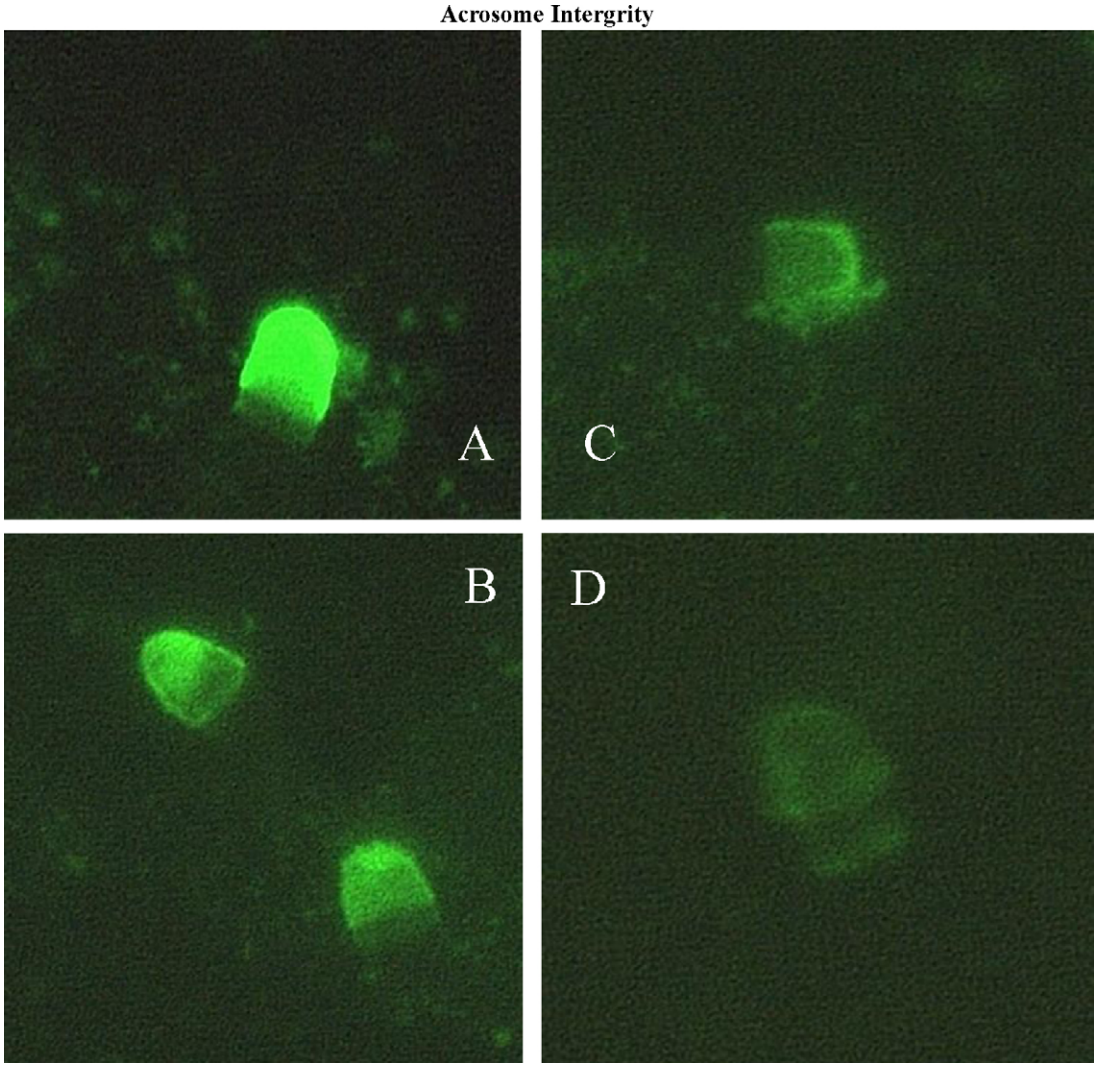

Fig. 3. Pictures illustrating the 4 categories of acrosome integrity evaluated in this study. (A) I-intact, (B) II-intermediate form of minimal reactivity, (C) III-intermediate form of severe reactivity and (D) IV-reacted acrosome.

transformed using the log base 10 (these values are given as log base 10 means ± S.E.M.) followed by Unpaired *t*-test. If the significant differences among the S.D. of the mean were still present after transformation, the Welch's correction was applied. The normality was confirmed by Kolmogorov–Smirnov test. Significant differences were evaluated based on two-tailed *p* values.

3. Results

3.1. Semen collection

Thirty-seven semen samples with motilities >50% were collected with a total volume of 29.86 ± 4.50 ml, a concentration of 164.53 ± 31.83 × 10^6 sperm/ml, a pH of 9.00 ± 0.20 a total motility of 82.58 ± 1.63%, progressive motility of 3.63 ± 1.03 and a SMI of 72.56 ± 2.96 (Table 1). No significant differences for the semen quality parameters were found between subspecies, therefore data was combined.

3.2. Split-sample comparison of directional and LN vapour freezing

Ejaculates with >50% total motility selected for this comparison did not show significant differences for motility, concentration, pH, volume, between subspecies and therefore data was combined. Average of raw data for split-sample comparison is reported as absolute values.

3.3. Directional freezing versus LN vapour freezing

DF improved the post-thaw quality of the pooled semen samples with respect to SMI by 8.1%, viability by 5.6% and progressive motility by 34.7% compared to LN (Fig. 5). No significant differences were found between normal morphology after DF and LN (Table 4).

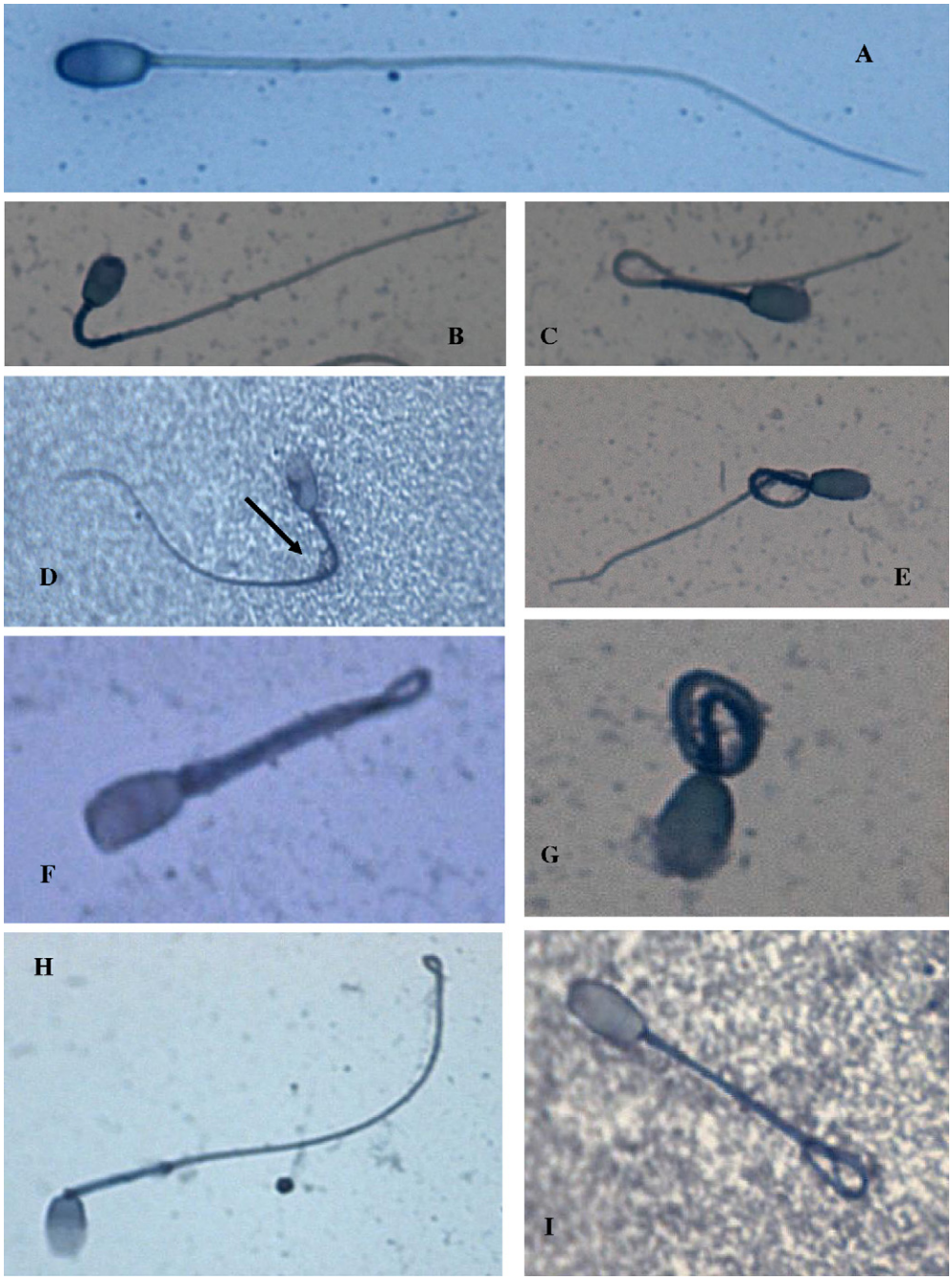

Fig. 4. Cell viability; hypo-osmotic swelling test. Subpart (A) indicates non-reacted and therefore non-viable spermatozoa, (B–I) indicate varying forms of reacted and therefore viable spermatozoan. Arrow indicates visible membrane and associated swelling.

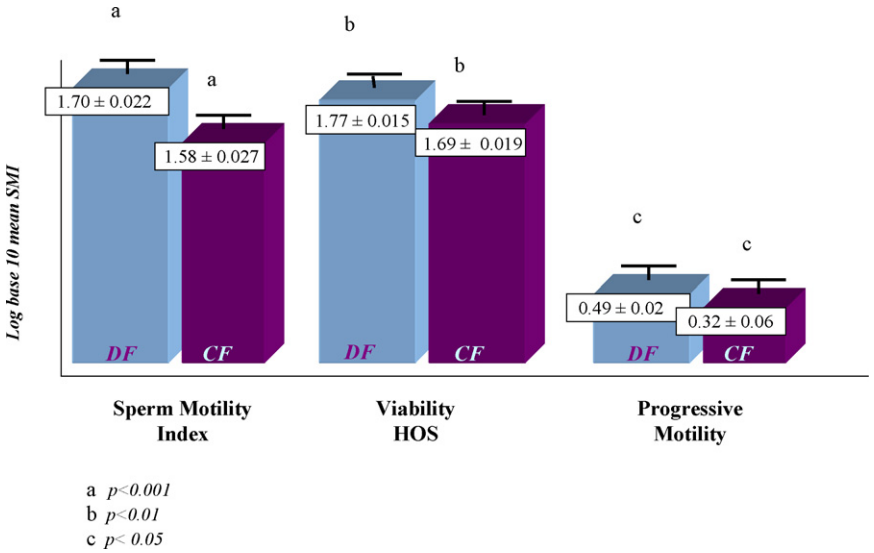

Fig. 5. Post-thaw results from the split-sample comparison of DF and LN freezing methods. Values are given as log base 10 means + S.E.M. Only parameters that demonstrated significant differences are illustrated. Values with like significant differences are indicated by common letters.

3.4. Effects of directional and LN vapour freezing on different pre-freeze sperm quality groups

After secondary assessment, the samples were divided into 3 categories based on pathomorphology (Table 3).

3.4.1. Low quality samples: <19% normal morphology

DF demonstrated 31.4% less abnormal acrosomes compared to LN ($p < 0.01$).

3.4.2. Moderate quality samples: 20–39% normal morphology

No statistical differences were found between DF and LN for the moderate quality semen group.

3.4.3. High quality samples: >40% normal morphology

DF demonstrated 18.7% improvement in intact acrosomes, 25% increase in progressive motility and subsequently a 10.9% improvement in SMI compared to LN.

Table 4
Post-thaw results of split-samples comparison of DF and LN

	Directional freezing	Conventional freezing	Statistical significance
Normal morphology	1.249 ± 0.07	1.16 ± 0.12	NS
Intact acrosomes	1.38 ± 0.11	1.12 ± 0.15	NS
No acrosomes	1.01 ± 0.09	1.04 ± 0.11	NS
Abnormal acrosomes	1.58 ± 0.04	1.50 ± 0.07	NS
Tail defects	1.03 ± 0.14	1.06 ± 0.12	NS
No tails	0.64 ± 0.08	0.80 ± 0.14	NS
Double defects	1.01 ± 0.09	1.24 ± 0.10	NS
HOS	1.77 ± 0.01	1.69 ± 0.01	$p < 0.01$
Total motility	1.55 ± 0.06	1.44 ± 0.07	NS
Progessive motility	0.49 ± 0.04	0.32 ± 0.06	$p < 0.05$
SMI	1.70 ± 0.02	1.58 ± 0.02	$p < 0.001$
TZI	1.24 ± 0.05	1.26 ± 0.56	NS
SDI	0.99 ± 0.05	1.03 ± 0.09	NS

Values are reported as log base 10 of the mean + standard error of the mean.

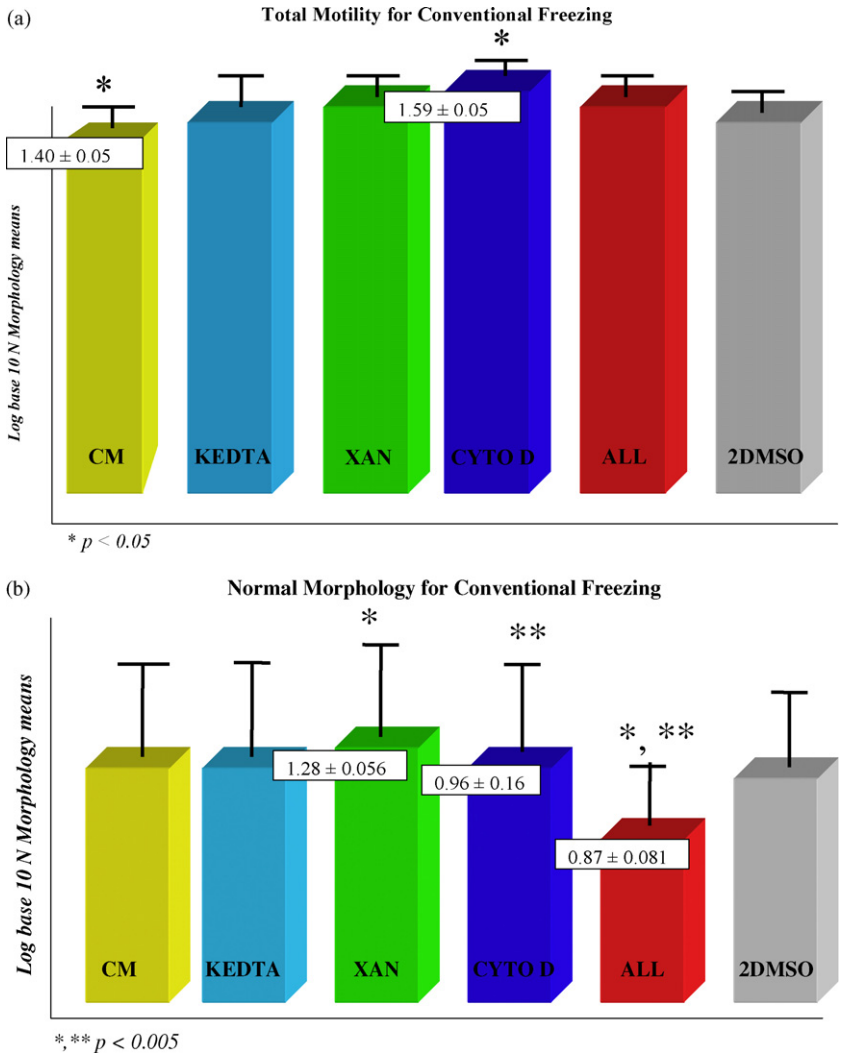

Fig. 6. (a) Post-thaw total motility results for extender experiment after LN. Values are given as log base 10 means + S.E.M. CYTOD improved sperm total motility by 17% compared to control medium after LN vapour freezing. (b) Post-thaw normal morphology results for extender experiment after LN. Values are given as log base 10 means + S.E.M. ALL dramatically decreased normal morphology by 36 and 31.1% compared to, respectively. Values with like significant differences are indicated by common symbols.

3.5. Extender additives

The extender KEDTA reduced the pre-freeze normal morphology after incubation by 14.4 and 17.4% compared to control medium and ALL, respectively ($p < 0.05$). After DF, the extenders control medium, KEDTA, CYTOD, XAN, ALL and 2DMSO dilution showed no statistically significant effects on post-thaw rhinoceros spermatozoal quality as defined by the parameters of conserved normal morphology, progressive and total motilities, viability/membrane function, and conserved acrosomal integrity.

After LN, CYTOD increased the total sperm motility by 17% compared to control medium, ($p < 0.05$) (Fig. 6a). ALL resulted in 36 and 31.1% lower conserved normal morphology compared to the XAN and CYTOD, respectively. ALL was significantly inferior to XAN and CYTOD ($p < 0.005$). XAN exhibited the highest absolute values for conserved normal morphology and ALL demonstrated the lowest (Fig. 6b). No significance difference for conservation of normal morphology was found among control medium, KEDTA and 2DMSO.

3.6. Calculation of putative fertility

Native fertility was calculated to be 62.4% based on the 43.67% pathomorphology. Cyropreservation

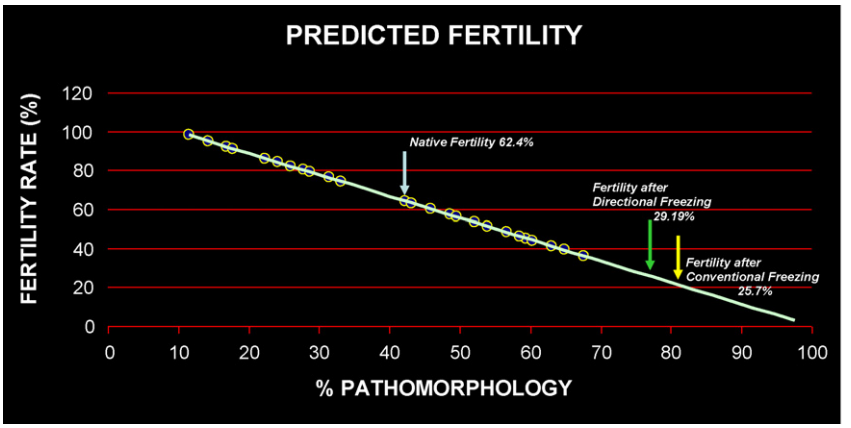

Fig. 7. Predicted fertility. Native fertility is predicated to be 62.4% based on equine fertility study (Pesch et al., 2006). Regression line $Y = -0.904x + 100.1$ ($r_s = -0.562$, $p < 0.001$) Directional freezing fertility is predicted to be 29.19% and LN vapour freezing 25.7%.

Table 5
Post-thaw spermatozoal head volumes after DF and LN of high quality semen group

	Meteromorphology (spermatoazoal head volume)
Native (pre-freeze)	116.4 ± 1.57[a]
Directional freezing	114.5 ± 2.87
Conventional freezing	109.8 ± 1.63[a]

[a] $p < 0.005$.

reduced the overall predicted sperm fertility by about 50%, however directional freezing with 78.4% pathomorphology and 29.19% fertility exhibited 3.49% higher calculated fertility compared to LN vapour freezing with 82.2% pathomorphology and 25.7% fertility (Fig. 7).

3.7. Spematozoal assessment

3.7.1. Effects of directional and LN vapour freezing on cell expansion

Pre-freeze average spermatozoa head volume was 11.64 ± 0.157 μm^2. After DF, the average sperm head volume decreased to 11.45 ± 0.287 μm^2. After LN, the average head volume decreased to 10.98 ± 0.163 μm^2. LN sperm head volume was 5.7% reduced compared to the pre-freeze or native head volume ($p < 0.005$). No significant differences in head volumes were found between native spermatozoa and spermatozoa cryopreserved using DF (Table 5).

4. Discussion

The quality of the samples collected in terms of concentration, volume, motility and percent structurally normal are within ranges reported in previous studies for white rhinoceroses [4,5,7].

After the general comparison of post-freezing semen quality parameters, directional freezing technology (DF) appeared superior to the LN vapour freezing technology (LN) in preserving the overall post-thaw functional plasma membrane integrity, progressive motility and consequently SMI of the rhinoceros spermatozoa. The DF results in this study coincided particularly with the results from another direct comparison of DF and LN in bulls, which reported increased post-thaw motility after large volume DF compared to LN and almost identical pregnancy rates after inseminated with large-volume, *double-frozen* semen using DF compared to that frozen *once* with LN [8]. Zirkler [23] demonstrated, however, no significant post-thaw differences between DF and LN in stallions. Although the DF freezing rate was identical, the protocol for LN differed from that used in this study. Perhaps, the differences in the LN technique could alone explain the discrepancy. This discrepancy could also be due to the overall species differences in freezability.

Other studies using DF in domestic species report similar results as found in this study. Gacitua and Arav [10], found no significant differences between pregnancy rates after double cervical inseminations using large volume buck semen frozen with DF compared with fresh semen. Si et al. (2006) reported total motility of $28.5 \pm 2.8\%$ and fertility rates of 73.9% for rabbit semen using the same extender as in this study.

LN results from other studies with rhinoceros semen were also comparable to the results from this study. Lubbe et al. [4,5] and Hermes et al. [7] reported similar post-thaw average total motility, which is expected

since the same LN technique was used. Higher SMI results after LN were reported for Sumatran and black rhinoceroses than in this study [6]. However, that study used different LN methods (dry shipper and dry ice), making it difficult to compare. In conclusion, DF improved the general post-thaw semen quality parameters of rhinoceros spermatozoa compared to LN. Further, large volumes of 2–10 ml were cryopreserved with DF at a higher post-thaw quality than that of LN in which small volumes of only 0.5–2 ml are possible in order to maintain an acceptable post-thaw quality in rhinoceroses (Blottner and Hermes, unpublished data). Volumes reflecting whole ejaculates (20–50 ml) currently used for the successful insemination of rhinoceroses [1]. Therefore, even without considering the increase in post-thaw quality resulting from DF, the fact that large volumes can be frozen while maintaining adequate post-thaw semen quality is logistically a substantial finding for the global implementation of ART.

Further, to evaluate the effects of the two freezing methods on varying semen quality states, three pre-freeze semen quality groups were formed based on normal and pathomorphology [24–28,17,12]. Although motility is the most accepted and commonly reported parameter for semen quality, pathomorphology and normal morphology were chosen because it has been shown to be more consistent across studies and verifiable even with varying assessment method [17]. The traditional parameter of motility is, in particular, inconsistent and not reliable for predicting semen quality [9,29–34]. This controversial discussion in stallions appears to relate to the fact that some subfertile and infertile animals maintain good progressive motility [17]. This phenomenon is also reflected by the rhinoceroses examined in this study. Consequently, morphology/pathomorphology was used as the determining factor for predicting semen quality. Significant differences where found for all the normospermic and teratozoospermic parameters among all three groups justifying the categorization.

The comparison of DF and LN effects on different pathomorphologic categories is based on the assumption that low quality pre-freeze semen is more sensitive to the freezing process (i.e. has a lower freezability). The cryopreservation of low quality semen (<19% normal morphology) was improved with DF in terms of a reduction in percent of abnormal acrosomes compared to LN. Zirkler [23] reported no differences between DF and LN for stallions with lower pre-freeze pathomorphology (‘‘good freezers’’, 23.1%) compared to those with higher pre-freeze pathomorphology (‘‘bad freezers’’, 39.5%); however, Zirkler [23] does report general superior post-thaw motility results for good freezers compared to bad freezers, further supporting the morphologic categorization. The fact that no significant differences were found between the two freezing methods in the moderate semen quality group (20–39% normal morphology) could be the result of over categorization. Zirkler [23], categorized stallion semen into only two groups: good freezability and bad freezability. High quality semen was improved with DF: increase in intact acrosomes and an improvement in SMI. In this study, DF demonstrated the ability to improve the cryopreservation of cryo-sensitive or compromised rhinoceros spermatozoa. Double-freezing studies using directional freezing have further illustrated this ability of DF to successfully cryopreserve already compromised spermatozoa while maintaining adequate fertilizing capacity [10,8,9].

The WHO (4th edition, 1999) [20] recommends that TZI values above 1.6 are associated with lower pregnancy rates and that an SDI value of 1.6 is the threshold for failure of fertilization *in vitro* based on a studies in humans ([35,36], respectively). In our study, we found SDI and TZI values greater than 1.6 only in the low quality group after LN. This may or may not be applicable for predicting fertilizing capacity of rhinoceros spermatozoa. However, this is an additional supporting finding for the conclusion that DF allows for the cyropreservation of low quality or already compromised spermatozoa. It also suggests that rhinoceros spermatozoa maintain a higher fertilization capacity when cyropreserved with DF.

An additional illustration of the different cellular effects of DF and LN methods on rhinoceros spermatozoa was demonstrated by volume regulation in response to the varying osmotic pressures during the cryopreservation process. Cell volume regulation was assessed as post-thaw spermatozoal head volume. After LN, the spermatozoal head volume was significantly reduced compared to pre-freeze, which was not significantly different to DF head volumes. The pre-freeze, DF and LN head volumes described in this study mimic those described as normal for rhinoceroses [37]. Osmotic stress results partly from the freezing of extracellular water at subzero temperatures producing increased osmolarity. Analogous changes occur during thawing. The ability of spermatozoa to respond to varying osmotic conditions and subsequently maintain normal spermatozoal head volume depends on the structural and functional plasma membrane integrity (as determined by the HOS test). The reduced maintenance of head volume was supported by the previous finding

of reduced functional plasma membrane integrity (HOS) after LN. Thus, LN appeared to inadequately maintain cellular membrane function resulting in reduced ability to maintain spermatozoal head volume in response to changing osmotic pressures potentially reducing fertilization capacity.

The maintenance of structural and functional integrity is greatly determined by the composition and properties of the plasma membrane [16]. Cryoprotective additives and cryomediums are employed to stabilize cells, reduce volume changes and balance osmotic stresses. Different additives with reported positive effects on post-thaw quality of stallion spermatozoa were added to the control medium to test for any positive or negative effects on rhinoceros spermatozoal survival.

The addition of EDTA was tested because of its function to chelate free calcium (Ca^{2+}) present in the cryomedium and naturally occurring in seminal plasma. Calcium is known to mediate capacitation and premature acrosomal reactivity and inhibit differential flip-flop movement of phospholipids [38–40]. Lipid peroxidation is a main contributor to the reduction of cellular integrity [41–44] and antioxidants are employed to reduce the damaging effects. In stallions, xanthurenic acid showed significantly higher percentage of progressive motility than other antioxidant tested [45]. Therefore, xanthurenic acid was substituted for alpha-tocopherol. Intracellular ice formation is another major contributing factor to cellular injury during cryopreservation. Previous studies suggest that the ability of the cell to expand to the required volume is more important in protecting cells from intracellular ice formation than controlling water permeability [39,46,47]. Cytochalasin D reversibly disrupts the F-actin filaments allowing for cell expansion without permanent damage to the cytoskeleton [48,49]. Potassium (K^+) is added to protect sodium–potassium (Na^+/K^+) pumps and to prevent the cellular depletion of K^+ during the reactivation of cellular metabolism in the thawing process [50].

After incubation, extender KEDTA reduced normal morphology compared to control medium and ALL, Potassium is positively charged and EDTA binds positively charged calcium molecules both of which alters the osmotic pressure of the extender environment for the spermatozoa. This may explain the pre-freeze effects seen.

DF appeared less sensitive to the extender composition than LN. No significant differences were found among the extenders control medium, KEDTA, CYTOD, XAN, ALL and 2DMSO dilution. After LN, however, positive effects of CYTOD resulted in an increase in total sperm motility compared to control medium, which could be a further example of the negative effects of LN on cell volume regulation. It seems that with DF the cell maintains the ability to regulate cellular volume in response to the osmotic pressures and therefore showed no measurable improvements with the addition of CYTOD. The extender ALL demonstrated the negative effects of lower conserved normal morphology compared to XAN and CYTOD. The difference in sensitivity to extender composition in general is more than likely due to the reduction in mechanical stress of DF.

In general, the extender additives tested did not exhibit the post-thaw increases in sperm quality parameters that were reportedly seen in the stallion. This is more than likely due to the fact that the previous studies comparing the individual additives in stallions did not always use an egg-yolk based extender. Egg-yolk has not only been shown to bind damaging seminal proteins and decrease the lipid-phase membrane temperature but also increases the efficacy of cytotoxic cryoprotective agents allowing for their reduction to concentration below the cytotoxic-threshold [9]. Egg-yolk seems to provide physiological components and an osmotic environment that is superior to non-egg-yolk cryomediums for the conservation of rhinoceros spermatozoa.

All these various above reported results of different semen quality parameters are difficult to interpret in regard to their collective or individual relationship to fertilizing capacity of the corresponding semen sample of rhinoceros bull. Fertility investigation in rhinoceros species are not possible due to the limited access to animals, the multi-factorial limited reproductive success of rhinoceros in captivity, particularly the influence of female reproductive pathology [51]. However, by carefully selecting a properly conducted fertility study, together with high-quality comprehensive reproductive information including not only spermatological and reproductive-ultrasound examinations but also information on multiple intrinsic and extrinsic factors such as genetic homogenicity, relationship to other group members especially females, housing and management, nutrition, health, number of and access to females, putative fertility can be generalized assuming that the intra-species variation may either be negligible or at least equally distributed. Fertility estimates should not be blindly accepted as fact or used alone to eliminate rhinoceros bulls from the breeding population. Having even a putative fertility together with a comprehensive reproductive assessment could further aid in evaluating

and isolating extrinsic factors that influence reproduction and fertility in male rhinoceroses in captivity. Further, it allows for a collective overview of the detailed multiple assessment results.

Putative fertility was calculated for the different freezing treatments based on fertility information from Pesch et al. [17]. Both freezing methods demonstrated an approximate 50% decrease in putative fertility compared to fresh semen. However, DF demonstrated a 3.49% increase compared to LN in putative fertility. This reflects findings in the literature that also suggest cryopreservation reduces fertility by at least half ([8,9,52] for review [46]) and that DF was less detrimental to fertility compared to LN [8]. This 3.49% difference between these two cryopreservation methods may seem trivial. However in terms of non-return rates (NR), each percent fertility increase represents one less mating/insemination attempt necessary for conception. For endangered wild species, where reproductive success is already limited and the risk of anesthesia is high, even this seemingly minimal increase is considered a substantial advance.

In short, using an egg-yolk based cryomedium and DF technology provided improved post-thaw spermatozoal quality in the rhinoceros compared to liquid nitrogen vapour conventional cryopreservation method. Further, results suggested the use of cytochalasin D when LN is to be employed. DF is further a promising method for the cryopreservation of other endangered wildlife species with highly cryo-sensitive spermatozoa.

Acknowledgements

We would like to express our gratitude for the financial support of this project provided by the Stipendium Stiftung des Abgeordentenhauses Berlins. Further, we would like to thank all the participating zoo and zoo staff for their cooperation on this project. Lastly, we sincerely thank the follow colleagues for their contributions that made this project possible: Andrea Krause, Britta Behr, Barbara Drews, Barbara Vogler, Kathleen Röllig, Dagmar Viertel and Angelika Kissman.

References

[1] Hildebrandt TB, Hermes R, Walzer C, Sós E, Molnar V, Mezösi L, et al. Artificial insemination in the anoestrous and the postpartum white rhinoceros using GnRH analogue to induce ovulation. Theriogenology 2007;67(9):1473–84.

[2] Roth TL. A review of the reproductive physiology of rhinoceros species in captivity. International zoo yearbook, vol. 40. 2006. p. 130–43.

[3] Platz C, Seager SWJ, Bush M. Collection and analysis of semen from a black rhinoceros. J Am Vet Med Assoc 1979;175:1002–4.

[4] Lubbe K, Smith RL, Bartels P, Godke RA. Freezing epididymal sperm from white rhinoceroses (*Ceratoterium simum*) treated with different cryodiluents. Theriogenology 1999;51:288.

[5] Lubbe K, Bartels P, Kilian I, Friedmann Y, Godke RA. Comparing motility and morphology of horse, zebra and rhinoceros epididymal spermatozoa when cryopreserved with two different cryodilutents or stored at 4 degrees Celsius. Theriogenology 2000;53:388.

[6] O'Brien JK, Roth TL. Post-coital sperm recovery and cryopreservation in Sumatran rhinoceroses (*Dicerorhinus sumatrensis*) and application of gamete rescue in the African black rhinoceroses (*Diceros bicornis*). J Reprod Fertil 2000;188:263–71.

[7] Hermes R, Hildebrandt TB, Blottner S, Walzer C, Silinski S, Patton ML, et al. Reproductive soundness of captive southern and northern white rhinoceroses (*Ceratoterium simum simum*, *C.s. cottoni*): evaluation of male genital tract morphology and semen quality before and after cryopreservation. Theriogenology 2005;63(1):219–38.

[8] Arav A, Zeron Y, Shturman H, Gacitua H. Successful pregnancies in cows following double freezing of a large volume of semen. Reprod Nutr Dev 2002;42(6):583–6.

[9] Si W, Hildebrandt TB, Reid C, Krieg R, Ji W, Fassbender M, et al. The successful double cryopreservation of rabbit (*Oryctolagus cuniculus*) semen in large volume using the directional freezing technique with reduced concentration of cryoprotectant. Theriogenology 2006;65(4):788–98.

[10] Gacitua H, Arav A. Successful pregnancies with directional freezing of large volume buck semen. Theriogenology 2005;63:931–8.

[11] Siema H, Arav A, Klus N, Klug E. Cryopreservation of stallion spermatozoa using a directional freezing technique. In: Proceedings of the second meeting of the European Equine Gamete Group (EEGG), September 2001, Loosdrecht, The Netherlands, Stout TAE and Wade JF (Eds). Havenmeyer Foundation Monograph Series No. 5. R &W Publications (Newmarket) Limited, 2002. pp. 17–19.

[12] Zirkler H, Gerbes K, Klug E, Sieme H. Cryopreservation of stallion semen collected from good and poor freezers using a directional freezing device (Harmony CryoCare—Multi Thermal Gradient 516). Anim Reprod Sci 2005;89(1–4):291–4.

[13] O'Brien JK, Robeck TR. Development of sperm sexing and associated reproductive technology for sex preselection of captive bottlenose dolphins (*Tursiops truncates*). Reprod Fertil Dev 2006;18:319–29.

[14] Pattie WA, Dowsett KF. The repeatability of seminal characteristics of stallions. J Reprod Fertil 1982;(Suppl. 32):9–13.

[15] Rousset H, Chanteloube P, Magistini M, Palmer E. Assessment of fertility and semen evaluations of stallions. J Reprod Fertil 1987;(Suppl. 35):25–31.

[16] Blottner S. Semen preservation for the management of reproduction in rare and endangered species. Adv Ethol 1998;(Suppl. 33):9–13.

[17] Pesch S, Bostedt H, Klaus F, Martin B. Advanced fertility diagnosis in stallion semen using transmission electron microscopy. Anim Reprod Sci 2006;91:285–98.

[18] He L, Bailey JL, Buhr MM. Incorporating lipids into boar sperm decreases chilling sensitivity but not capacitation potential. Biol Reprod 2001;64:69–79.

[19] Huang CH. Studies on phosphatidylcholine vesicles: formation and physical characteristics. Biochemistry 1969;8:334–52.

[20] World Health Organization (WHO). Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction, 4th edn., UK: Cambridge University Press, Cambridge; 1999.
[21] Blottner S, Fasinski M, Pitra Ch. Charakterisierung der in-vitro-kapazitation motiler Bullen-spermien durch die Hyamin-induzierte Akrosomenreaktion. Arch Exp Vet Med 1989;43:285–92.
[22] Van der Ven HH, Jeyendran RS, Al-Hassani S, Perez-Pelaez M, Diedrich K, Zaneveld LJD. Correlation between human sperm swelling in hypoosmotic medium (hypoosmotic swelling test) and in vitro fertilization. J Androl 1986;111:190–6.
[23] Zirkler H. Kryokonservierung von Hengstsperma mittels direktionaler Gefriertechnik in Hollow-tubes im Vergleich zur konventionellen Gefrierung in 0.5 ml Pailletten. Thesis. Die Stiftung Tieraertzliche Hochschule Hannover, Germany; 2005 [in German with an English abstract].
[24] Jasko DJ, Lein DH, Footie RH. Determination of the relationship between sperm morphologic classifications and fertiliy in stallions: 66 cases (1987–1988). JAVMA 1990;197(3):389–94.
[25] Jasko DJ, Little TV, Lein DH, Footie RH. Determination of stallion semen quality and its relationship with fertility. J Reprod Fertil 1991;(Suppl. 44):649–50.
[26] Jasko DJ, Little TV, Lein DH, Footie RH. Comparison of spermatozoal movement and semen characteristics with fertility in stallions: 64 cases (1987–1988). JAVMA 1992;200(7): 979–85.
[27] Chevalier-Clement F, Hochereau de Reviers MT, Perreau C, Magistrini M. Alterations of the semen and genital tract of infertile stallions. J Reprod Fertil 1991;(Suppl. 4):657–8.
[28] Bader H, Gremmes S, Sieme H, Paar M, Hoppen H, Brandt K. Subfertility in the male Przewalski horse. J Reprod Fertil 1991;11:133–8.
[29] Hanada A, Nagase H. Cryoprotective effects of some amides on rabbit spermatozoa. J Reprod Fertil 1980;60:247–52.
[30] Mann T. Biochemistry of semen and of the male reproductive tract. London/New York: Methuen & Co. Ltd./John Wiley & Sons, Inc.; 1964. p. 1–347.
[31] Rodriguez-Martinez H. Laboratory semen assessment and prediction of fertility: still utopia? Reprod Domest Anim 2003;38: 312–8.
[32] Dowsett KF, Pattie WA. Characteristics and fertility of stallion semen. J Reprod Fertil 1982;(Suppl. 32):1–8.
[33] Boyle MS. Artificial insemination: assessing stallion semen quality after freezing. J Equine Vet Sci 1996;28:5–6.
[34] Grondahl C, Grondahl ML, Hyttel P, Greve T. Acosomal status in fresh and frozen/thawed stallion spermatozoa evaluated by scanning electron microscopy. Anat Embryol 1994;190:195–200.
[35] Jouannet P, Ducot B, Feneux D, Spira A. Male factors and the likelihood of pregnancy in infertile couples. I. Study of sperm characteristics. Int J Androl 1988;11:379–94.
[36] Aziz N, Buchan I, Taylor C, Kingland CR, Lewis-Jones I. The sperm deformity index: a reliable predictor of the outcome of oocyte fertilization in vitro. Fertil Steril 1996;66:1000–8.
[37] Silinski S. Pharmacologically enhanced manual semen collection and computer assisted sperm head morphology in the white rhinoceros (*Ceratotherium simum*). Thesis. Ludwig-Maximilian-University, Munich, Germany; 2003 [in German with English abstract].
[38] Bedford S, Verner DD, Meyers SA. Effects of cryopreservation on the acrosomal status of stallion spermatozoa. Theriogenology 2000;56:133–40.
[39] Watson PF. The causes of reduced fertility with cyropreserved semen. Anim Reprod Sci 2000;60–61:481–92.
[40] James PS, Wolfe CA, Mackie A, Ladha S, Prentice A, Jones R. Lipid dynamics in the plasma membrane of fresh and cryopreserved human spermatozoa. Hum Reprod 1999;14(7):1827–32.
[41] De Lamirande E, Gagnon C. Reactive oxygen species and human spermatozoa. I. Effects on the motility of intact spermatozoa and on sperm axonemes. J Androl 1992;13:368–78.
[42] Aitken RJ, Buckingham DW, Harkiss D. Use of a xanthine oxidase free radical gathering system to investigate the cytotoxic effects of reactive oxygen species on human spermatozoa. J Reprod Fertil 1993;97:441–50.
[43] Aitken RJ. Free radicals, lipid peroxidation and sperm function. J Reprod Fertil 1995;7:659–68.
[44] Griveau JF, Dumont E, Renard P, Callegari JP, LeLannou D. Reactive oxygen species, lipid peroxidation and enzymatic defense systems in human spermatozoa. J Reprod Fertil 1995;103:17–26.
[45] Denniston DJ, Squires EL, Bruemmer JE, Brinsko SP, McCue PM, Graham JK. Effect of antioxidants on the motility and viability of cooled stallion spermatozoa. J Reprod Fertil 2000;(Suppl. 56):121–6.
[46] Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. J Reprod Fertil 1995;7:871–91.
[47] Petrunkina AM, Groepper B, Toeper-Petersen E, Guenzel-Apel A-R. Volume regulatory function and sperm membrane dynamics as parameters for evaluating cryoprotective efficiency of a freezing extender. Theriogenology 2005;63:1390–406.
[48] Noiles EE, Bailey JL, Storey BT. Water permeability, Lp, of the mouse sperm plasma membrane and its activation energy are strongly dependent on interaction of the plasma membrane with the sperm cytoskeleton. Cryobiology 1997;35:79–92.
[49] Holt WV, Penfold LM, Johnston SD, Temple-Smith P, McCallum C, Shaw J, et al. Cryopreservation of macropodid spermatozoa: new insights from the cryomicroscope. J Reprod Fertil 1999;11(6):345–53.
[50] Gadea J. Pig industry—semen extenders used in the artificial insemination of swine. A review. Spanish J Agric Res 2003;1(2): 17–27.
[51] Hermes R, Hildebrandt TB, Walzer C, Göritz F, Patton ML, Silinski S, et al. The effect of long non-reproductive periods on the genital health in captive female white rhinoceroses (*Ceratotherium simum simum*, *C.s. cottoni*). Theriogenology 2006;65(8):1492–515.
[52] Rasul Z, Ahmad N, Anzar M. Changes in motion characteristics, plasma membrane integrity, and acrosome morphology during cryopreservation of buffalo spermatozoa. J Androl 2001;22(2): 278–83.