Available online at www.sciencedirect.com
ScienceDirect
Theriogenology 71 (2009) 393–399
Theriogenology
www.theriojournal.com

First successful artificial insemination with frozen-thawed semen in rhinoceros

R. Hermes [a,*], F. Göritz [a], J. Saragusty [a], E. Sós [b], V. Molnar [b], C.E. Reid [a], F. Schwarzenberger [c], T.B. Hildebrandt [a]

[a] *Leibniz Institute for Zoo and Wildlife research, Alfred-Kowalke-Straße 17, D-10315 Berlin, Germany*
[b] *Zoo Budapest, Állatkerti krt. 6-12, H-1146 Budapest, Hungary*
[c] *Department of Biomedical Sciences (Biochemistry), University of Veterinary Medicine, A-1210 Vienna, Austria*

Received 29 September 2008; received in revised form 22 October 2008; accepted 22 October 2008

Abstract

The first successful artificial insemination (AI) in a rhinoceros was reported in 2007 using fresh semen. Following that success, we decided to evaluate the possibility of using frozen-thawed semen for artificial insemination. Semen, collected from a 35–36 year old Southern white rhinoceros (*Ceratotherium simum simum*) in the UK was frozen using the directional freezing technique. This frozen semen was used in two intrauterine AI attempts on a 30 years old female rhinoceros in Hungary. The first attempt, conducted 30 days postpartum with an insemination dose of $\sim 135 \times 10^6$ motile cells, failed. The second attempt, conducted two estrus cycles later with an insemination dose of $\sim 500 \times 10^6$ motile cells, resulted in pregnancy and the birth of a healthy offspring. This represents the first successful AI using frozen-thawed semen in a rhinoceros, putting it among very few wildlife species in which AI with frozen-thawed semen resulted in a live birth. The incorporation of AI with frozen-thawed semen into the assisted reproduction toolbox opens the way to preserve and transport semen between distant individuals in captivity or between wild and captive populations, without the need to transport stressed or potentially disease carrying animals. In addition, cryopreserved spermatozoa, in combination with AI, are useful methods to extend the reproductive lifespan of individuals beyond their biological lifespan and an important tool for managing genetic diversity in these endangered mammals.
© 2009 Elsevier Inc. All rights reserved.

Keywords: Genome bank; Reproduction; White rhinoceros; Directional freezing; Cryopreservation; *Ceratotherium simum simum*

1. Introduction

As of 2006, the worldwide rhinoceros population constituted about 14,950 animals in Africa, 2850 in Asia and approximately 800 in captivity [1]. For many years, the number of rhinoceros species has declined significantly due to poaching, hunting, habitat degradation and fragmentation, as well as civil wars in range countries. Although recent reports indicate some success in conservation efforts [2], two of the three species in Asia and three of the six subspecies in Africa are still listed as critically endangered [3]. One of these, the Northern white rhinoceros, has an estimated four individuals left in the wild. According to recent reports, it is unclear whether these animals are still alive as they have not been seen since 2006 [2]. An additional African subspecies, the Western black rhinoceros, is thought to be extinct [4]. Although it is not understood why, the reproductive success of rhinoceros in captivity is low, especially among those born in captivity [5]. As a

* Corresponding author at: Department of Reproduction Management, Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Straße 17, D-10315 Berlin, Germany.
Tel.: +49 30 5168 243 fax: +49 30 5126 104.
E-mail address: hermes@izw-berlin.de (R. Hermes).

0093-691X/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2008.10.008

result, the rhinoceros has a projected negative annual growth rate of 3.5% [6]. With this bleak forecast in hand, extensive efforts were invested in recent years to understand the reproductive anatomy and biology of the various rhinoceros species [7–14]. These efforts were supplemented by the development and extensive use of ultrasonographic reproductive assessments in captive animals [15–18]. These evaluations led to the understanding that aging of the captive female population as well as repeated, non-reproductive oestrus cycles are major contributors to the development of a variety of reproductive pathologies [16,19]. In male specimens, ultrasonographic evaluations of the reproductive tract enabled the development of successful semen collection and cryopreservation techniques [15,20]. These advances have allowed the development of an artificial insemination technique for rhinoceros [21]. This technique has been modified and improved over the years, a special intrauterine insemination catheter was developed and a protocol to induce ovulation was devised, all of which contributed to the birth of the world's first live rhinoceros calf conceived by artificial insemination at the beginning of 2007 [22]. As important as this achievement was, the use of fresh semen collected from a male in the same institution limited wider application of the technique.

Initial sperm cryopreservation experiments using the equiaxed liquid nitrogen vapor method resulted in relatively poor post-thaw survival [15,23]. However, another freezing technique known as directional freezing offered a new opportunity. This technique makes it possible to precisely control ice crystal propagation, morphology and latent heat dissipation when freezing in large volumes [24]. Several attempts using this technique in a variety of species have proved it to be successful [25–28] and superior to conventional freezing techniques, notably in stallions which are often used as model species for the development of analogous protocols in rhinoceros [29,30]. Using this technique, we showed recently that *in vitro* post-thaw rhino semen parameters can be significantly enhanced in comparison to the conventional, liquid nitrogen vapor technique [31]. With these encouraging results, the next step was to attempt artificial insemination with frozen-thawed semen in rhinoceros.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all materials were obtained from Sigma Aldrich Chemie GmbH, Taufkirchen, Germany.

2.2. Animals

One wild caught female and one non-proven wild caught male Southern white rhinoceros (*Ceratotherium simum simum*; studbook # 902 and # 88, respectively) participated in this study. The female's previous two pregnancies were conceived through artificial insemination with fresh semen [22]. At the time of conception, the female was housed at the Budapest Zoo in Hungary and the male in Colchester Zoo in the UK. The male was 35–36 years old at the time of semen collections and the female was 30 at the time of insemination using the frozen-thawed semen.

2.3. Endocrinology

The female's endocrine function was monitored by serum and fecal progesterone metabolite analysis at least on a bi-weekly basis since it gave birth in January, 2007. For collection of blood samples, the female rhinoceros was conditioned to accept blood withdrawal from an ear vein. Freshly defecated faeces were collected in the stalls. Plasma and fecal samples were stored at −20 °C pending analysis, which was conducted using a group-specific enzyme-immunoassay for 20-oxo pregnanes as described previously [22,32]. Briefly, before analysis plasma samples were extracted with diethyl ether and faecal samples were extracted with methanol and petroleum ether. The extracts were diluted with assay buffer depending on concentrations.

To induce and time ovulation in accordance with the planned AI, 4.2 mg synthetic GnRH analogue, deslorelin acetate (Ovuplant™, Peptech, Melbourne, Australia) were administered by a biocompatible, sustained release subcutaneous implant as previously described [22].

2.4. Anaesthesia

Standing sedation was used for transrectal ultrasonographic examinations, for AI and for pregnancy determination. This was achieved by injecting 25.0 mg detomidine hydrochloride (Domosedan®, Orion Corporation, Espoo, Finland) and 25.0 mg butorphanol (Butormidor®, Richter Pharma AG, Wels, Austria). Due to the short period between anaesthesia, sedation of the female for artificial insemination required the additional application of etorphine hydrochloride (0.98–1.72 mg) and acepromazine maleate (4–7 mg) (Large Animal Immobilon®, Novartis Animal Health UK Ltd., Royston, UK) and 200 mg ketamine (Bela

Pharm GmbH & Co. KG, Vechta, Germany). Local anaesthetic lidocaine-hydrochloride Gel (Xylocain® Gel 2%, AstraZeneca GmbH, Wedel, Germany) was applied to the vaginal labia to avoid reflex reactions when manipulating the clitoris during insemination.

Electro-ejaculation required general anaesthesia which was achieved with 15.0 mg detomidine hydrochloride and 15.0 mg butorphanol. This was followed by 3.06 mg etorphine hydrochloride and 12.5 mg acepromazine maleate. In addition, 150 mg ketamine were injected into the ear vein. For reversal, 200–250 mg naltrexone hydrochloride (Wildlife Pharmaceuticals, Fort Collins, CO) was administered intra venous This was accompanied by intra venous application of 100–125 mg atipamezole (Antisedan®, Orion Corporation, Espoo, Finland) in cases when Large Animal Immobilon® was used [19].

2.5. Semen collection and cryopreservation

Semen was collected on two occasions using electro-ejaculation (Seager model 14, Dalzell USA Medical Systems, The Plains, VA) as previously described [15]. Briefly, a specially designed probe, 125 mm long with a diameter of 105 mm and three longitudinal, slightly raised electrodes, was introduced into the male's rectum. On each occasion, a total of 18 stimulations, divided into sets of 3–4 stimulations, were applied with increasing voltage (range 3–15 V) and amperage (maximum 500 mA). After collection, the total volume was noted and the semen was immediately extended with isothermal Berliner Cryomedium (BC) [15,33], a TEST-egg yolk extender with 6.25% Me_2SO, at a ratio of 1:1. Subjective progressive motility on a scale of 0 to 5 (0 = immotile, 5 = fast progressive motility) and total motility of the extended native semen was evaluated by dark field microscope (Olympus CH 40, Olympus, Hamburg, Germany) equipped with a heating stage (37 °C). Sperm concentration was estimated using an improved Neubauer haemocytometer. Smears were prepared for later evaluation of sperm morphology and acrosome integrity and an aliquot of native sperm was incubated for a hypoosmotic swelling (HOS) test as previously reported [31].

The extended semen was chilled slowly over ~2 h by placing the test tube with the semen inside an isothermal water bath stored at 4 °C. Following chilling, the semen was packaged into 8 mL and 2.5 mL HollowTubes and frozen using the MTG-516 apparatus (IMT Ltd., Nes Ziona, Israel) as previously described [26,27,30,31]. Frozen samples were kept under liquid nitrogen pending insemination. For thawing, samples were first held in the air at room temperature (22–23 °C) for 60 s and then plunged into a water bath at 37 °C for 30 s. Following thawing, samples were kept in the water bath at 37 °C pending evaluation and artificial insemination.

2.6. Artificial insemination and pregnancy confirmation

Several days prior to insemination, a thorough ultrasound examination of the reproductive tract was conducted (Voluson *i*, GE Medical Systems, Zipf, Austria, with 2–5 MHz and 4–8.5 MHz transducers or SonoSite 180Plus, SonoSite Inc., Borthell, WA, with 2–5 MHz C60/5-2 transducer). On each occasion, a single insemination was performed in the sedated female with 16 mL of extended sperm which was thawed just prior to the procedure. For insemination, a special catheter was designed to overcome the two main anatomical obstacles for intrauterine insemination in the rhinoceros. These obstacles are the hymeneal membrane in nulliparous females and the anatomically long and tortuous rhinoceros cervix, which has extremely tight cervical folds. This 115 cm long catheter is composed on the outside of a conic (4–9 mm) flexible carbon sheath with a handle. On the inside, the carbon sheath holds a 90 cm luer-lock cannula, which ends in an angled, smoothly edged stainless steel catheter tip (Patent: DE 10203094A1, Schnorrenberg Chirurgiemechanik GmbH, Woltersdorf, Germany).

Pregnancy diagnosis and foetal development were evaluated in the standing sedated animal with the aid of ultrasound. Foetal measurements of different biometric parameters included: crown-to-rump length, biparietal width, thoracic width and femur length. Biometric measurements of the fetus were extracted directly from on board stored scans and processed with 4D View software (GE Medical Systems).

3. Results

Two inseminations were performed. The first was conducted during the postpartum heat, 28 days postpartum. This AI failed to result in a pregnancy as was indicated by the appearance of the following oestrus cycle. A second attempt was carried out during the third postpartum cycle. In this attempt, a pregnancy was achieved as a result of artificial insemination with frozen-thawed semen and a live male calf was born.

3.1. Semen collection and cryopreservation

The semen was collected on two occasions from an aged male who never sired offspring. On the two

Table 1
Fresh and post-thaw sperm parameters.

	First AI		Second AI	
	Fresh	Frozen-thawed	Fresh	Frozen-thawed
Concentration ($\times 10^6$ cells/mL)	80	40	80	40
Total motility (%)	90	21	90	78
Progressive motility (0–5 scale)	3	2	5	5
Normal morphology (%)	71	25	76	61
Intact acrosome (%)	91	30	76	65
Viability (HOS positive) (%)	79	74	70	48
Total cells for AI ($\times 10^6$)		640		640
Total motile cells for AI ($\times 10^6$)		135		500

occasions, a total of 56 mL and 27.5 mL were collected in 5 and 3 fractions, respectively. Fresh and post-thaw sperm evaluations indicated good to moderate quality sperm (Table 1). The semen was frozen and kept under liquid nitrogen for 2–3 years before it was used for the AI. For each AI, 2 × 8 mL tubes were thawed. Both insemination doses contained a total of ~640 × 10^6 cells. However, the first insemination was performed with about 135 × 10^6 motile cells while the second with about 500 × 10^6 motile cells.

3.2. Artificial insemination and pregnancy confirmation

During ultrasound evaluations conducted 1–4 days prior to AI, a Graafian follicle was identified with a diameter of 3.4 and 2.65 cm for the first and second AI, respectively. Administration of the GnRH analogue in both attempts resulted in ovulation within 24 h. This was confirmed by the appearance of corpus haemorrhagicum, Ø 3.5 and Ø 3.4 cm on the first and second AI, respectively. Under ultrasonographic guidance, the specially designed insemination catheter was directed through the cervix and the uterine body into the uterine horn on the ovulating side.

Serum pregnane concentrations rose and remained elevated after the second AI. However, fecal pregnane concentrations were slower to raise and did not indicate gestation until 4–5 months after the insemination (Fig. 1). Pregnancy was confirmed by ultrasound on day 109 of gestation and the fetal measurements of the different biometric parameters were: biparietal width 3.08 cm, thorax width 5.23 cm, crown-to-rump length 15.23 cm and femur length 2.04 cm. A healthy male calf was born after 495 days of gestation (Fig. 2).

4. Discussion

In this study we used frozen-thawed semen collected from an aged male Southern white rhinoceros that had never sired offspring, and was therefore not represented in the population, to inseminate a female housed at a

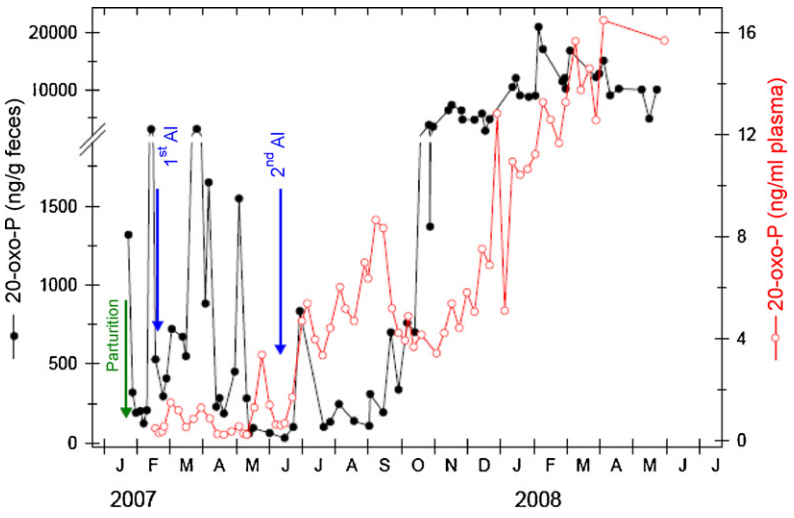

Fig. 1. Serum and fecal 20-oxo-pregnane from last parturition to the 12th month of the pregnancy following AI with frozen-thawed semen.

Fig. 2. The world's first rhinoceros to be born following artificial insemination with frozen-thawed semen, several hours after birth.

distant institution. During the first AI we unsuccessfully attempted to ''catch'' the foal heat. This failure might have been due to insufficient sperm quality in terms of low post thaw motility and low levels of normal morphology and acrosome integrity. The quantity of motile cells (approximately 135×10^6) may have been below the minimum threshold required to achieve fertilization in this species. It might also be that the low post thaw motility in this sample was due to extensive DNA fragmentation, which seem to be a frequent problem in frozen-thawed rhinoceros semen (unpublished data). The second, successful attempt which took place during the third postpartum cycle used approximately 500×10^6 motile cells.

This achievement joins a fairly short list of fewer than 30 wildlife species, most of which closely related to domestic species, in which artificial insemination with frozen-thawed semen has been successful in producing live offspring (For reviews see: [35,36–38]). The use of frozen-thawed semen holds a great potential as a mean to help overcome the crisis most captive and wild rhinoceros populations are facing in many different ways.

As was demonstrated here and in our previous study [22], artificial insemination with both fresh and cryopreserved semen is now possible in the rhinoceros. This very important reproductive technique can now be incorporated into the rhinoceros conservation toolbox and is expected to enhance the poor reproductive performance of the captive rhinoceros populations. It may also help in saving the Northern white rhinoceros subspecies from extinction. For other rhinoceros species and subspecies, semen can also be collected in the wild to increase the genetic diversity of the captive population without the need to remove individuals from their habitat and without the risk of transferring potentially disease-carrying animals between countries. Frozen semen can be stored while blood samples are evaluated to ascertain that it carries no transmissible disease before using it for insemination. Collection and freezing of rhinoceros semen also allows samples to be collected from captive males and used to invigorate small and isolated populations in the wild. The possibility to move semen between distant institutions for the purpose of AI, as demonstrated in this study, should also help to reverse the premature reproductive aging due to the development of reproductive tract pathologies and the secession of ovarian activity [16,19].

As if to worsen the already poor reproductive record of the various rhinoceros species and subspecies in captivity, reports on both the Indian (greater one-horned) and the black rhinoceroses indicate that the captive birth sex ratio is skewed towards males. In the Indian rhinoceros the reported zoo-born sex ratio is 60:40 (male:female) [39]. In the wild-caught black rhinoceros population it was reported that the odds of having a male offspring increase several fold with the age of the dam or with the duration that the female is held in captivity [40] or if the female is translocated just prior to conception or during early pregnancy [41]. We have recently demonstrated the possibility to sort the

rhinoceros sperm into X- and Y-chromosome bearing sperm populations with the aid of flow cytometry [42]. Combining this technique with sperm freezing and artificial insemination may help not only to counter skewed sex ratio trends, but also through insemination with X-chromosome bearing spermatozoa to increase the proportion of females in the population and thus to speed-up offspring production.

It was previously reported in the black, white, Indian and Sumatran rhinoceros species that, while fecal progesterone metabolites are reliable indicators of pregnancy, as fecal steroid metabolites rise substantially over and above the level of the luteal phase within the first 3–5 months of gestation [7–11,13,14,18,22,34]. This pattern was confirmed in the present case (Fig. 1). However, fecal pregnane metabolites did not closely reflect the elevated serum progesterone levels during the first 4–5 months of pregnancy and therefore may not be accurate for determining early pregnancy. The reasons for this inconsistency in plasma and fecal hormone levels after frozen-thawed semen are not clear, as during two previous gestations in this same animal fecal hormones clearly increased already after the second month of pregnancy [22]. This is also true for steroid metabolites measured over the course of pregnancy following natural mating [8,9,32].

In conclusion, the world's first successful artificial insemination with frozen-thawed semen in rhinoceros holds great promise for the future of these mega-herbivores. Semen samples can be collected and preserved from both wild and captive populations to maintain a genome resource bank and to boost reproduction in these endangered mammals.

Acknowledgements

The authors wish to thank Mr. Bela Szandelszky for kindly providing us with the photograph of the mother and calf.

References

[1] Amin R, Thomas K, Emslie RH, Foose TJ, Van Strien N. An overview of the conservation status of and threats to rhinoceros species in the wild. Int Zoo Yearbook 2006;40:96–117.
[2] IUCN. Rhinos on the rise in Africa but Northern white nears extinction. Gland, Switzerland (2008; Press release).
[3] IUCN. IUCN Red List of Threatened Species. Gland, Switzerland: International Union for the Conservation of Nature, 2007.
[4] Anonymous. West African black rhino feared extinct. IUCN Newslett Species Surviv Comm 2006;46:31.
[5] Swaisgood RR, Dickman DM, White AM. A captive population in crisis: testing hypotheses for reproductive failure in captive-born southern white rhinoceros females. Biol Conserv 2006;129: 468–76.
[6] Emslie R, Brooks M. African rhino: status survey and conservation action plan. Gland, Switzerland and Cambridge, UK: IUCN; 1999, ix+92.
[7] Heistermann M, Agil M, Buthe A, Hodges JK. Metabolism and excretion of oestradiol-17β and progesterone in the Sumatran rhinoceros (*Dicerorhinus sumatrensis*). Anim Reprod Sci 1998;53:157–72.
[8] Patton ML, Swaisgood RR, Czekala NM, White AM, Fetter GA, Montagne JP, et al. Reproductive cycle length and pregnancy in the southern white rhinoceros (*Ceratotherium simum simum*) as determined by fecal pregnane analysis and observations of mating behavior. Zoo Biol 1999;18:111–27.
[9] Radcliffe RW, Czekala NM, Osofsky SA. Combined serial ultrasonography and fecal progestin analysis for reproductive evaluation of the female white rhinoceros (*Ceratotherium simum simum*): preliminary results. Zoo Biol 1997;16:445–56.
[10] Schwarzenberger F, Tomášová K, Holecková D, Matern B, Möstl E. Measurement of fecal steroids in the black rhinoceros (*Diceros bicornis*) using group-specific enzyme immunoassays for 20-oxo-pregnanes. Zoo Biol 1996;15:159–71.
[11] Stoops MA, Pairan RD, Roth TL. Follicular, endocrine and behavioural dynamics of the Indian rhinoceros (*Rhinoceros unicornis*) oestrous cycle. Reproduction 2004;128:843–56.
[12] Godfrey RW, Pope CE, Dresser BL, Olsen JH. Gross anatomy of the reproductive tract of female black (*Diceros bicornis michaeli*) and white rhinoceros (*Ceratotherium simum simum*). Zoo Biol 1991;10:165–75.
[13] Schwarzenberger F, Rietschel W, Vahala J, Holeckova D, Thomas P, Maltzan J, et al. Fecal progesterone, estrogen, and androgen metabolites for noninvasive monitoring of reproductive function in the female Indian rhinoceros, *Rhinoceros unicornis*. Gen Comp Endocrinol 2000;119:300–7.
[14] Schwarzenberger F, Francke R, Goltenboth R. Concentrations of faecal immunoreactive progestagen metabolites during the oestrous cycle and pregnancy in the black rhinoceros (*Diceros bicornis michaeli*). J Reprod Fertil 1993;98:285–91.
[15] Hermes R, Hildebrandt TB, Blottner S, Walzer C, Silinski S, Patton ML, et al. Reproductive soundness of captive southern and northern white rhinoceroses (*Ceratotherium simum simum*, C.s. cottoni): evaluation of male genital tract morphology and semen quality before and after cryopreservation. Theriogenology 2005;63:219–38.
[16] Hermes R, Hildebrandt TB, Göritz F. Reproductive problems directly attributable to long-term captivity-asymmetric reproductive aging. Anim Reprod Sci 2004;82–83:49–60.
[17] Hildebrandt TB, Göritz F, Hermes R. Ultrasonography: an important tool in captive breeding management in elephants and rhinoceroses. Eur J Wildl Res 2006;52:23–7.
[18] Roth TL, Bateman HL, Kroll JL, Steinetz BG, Reinhart PR. Endocrine and ultrasonographic characterization of a successful pregnancy in a Sumatran rhinoceros (*Dicerorhinus sumatrensis)* supplemented with a synthetic progestin. Zoo Biol 2004;23: 219–38.
[19] 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: 1492–515.
[20] Roth TL, Stoops MA, Atkinson MW, Blumer ES, Campbell MK, Cameron KN, et al. Semen collection in rhinoceroses (*Rhinoceros*

unicornis, Diceros bicornis, Ceratotherium simum) by electroejaculation with a uniquely designed probe. J Zoo Wildl Med 2005;36:617–27.

[21] Hildebrandt TB, Hermes R, Schwarzenberger F, Walzer C, Silinski S, Schnorrenberg A, et al. A newly developed artificial insemination technique in African rhinoceroses. In: Proceedings of the American Association of Zoo Veterinarians; 2002. p. 213–5.

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

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

[24] Arav A. Device and methods for multigradient directional cooling and warming of biological samples. US Patent: Arav, Amir, 1999.

[25] 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;65:788–98.

[26] Saragusty J, Gacitua H, King R, Arav A. Post-mortem semen cryopreservation and characterization in two different endangered gazelle species (*Gazella gazella* and *Gazella dorcas*) and one subspecies (*Gazella gazelle acaiae*). Theriogenology 2006;66:775–84.

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

[28] 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;42:583–6.

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

[30] Saragusty J, Gacitua H, Pettit MT, Arav A. Directional freezing of equine semen in large volumes. Reprod Domest Anim 2007;42:610–5.

[31] Reid CE, Hermes R, Blottner S, Goeritz F, Wibbelt G, Walzer C et al. 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*). Theriogenology, in press.

[32] Schwarzenberger F, Walzer C, Tomasova K, Vahala J, Meister J, Goodrowe KL, et al. Faecal progesterone metabolite analysis for non-invasive monitoring of reproductive function in the white rhinoceros (*Ceratotherium simum*). Anim Reprod Sci 1998;53:173–90.

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

[34] Garnier JN, Holt WV, Watson PF. Non-invasive assessment of oestrous cycles and evaluation of reproductive seasonality in the female wild black rhinoceros (*Diceros bicornis minor*). Reproduction 2002;123:877–89.

[35] Andrabi SMH, Maxwell WMC. A review on reproductive biotechnologies for conservation of endangered mammalian species. Anim Reprod Sci 2007;99:223–43.

[36] Pukazhenthi BS, Wildt DE. Which reproductive technologies are most relevant to studying, managing and conserving wildlife? Reprod Fertil Dev 2004;16:14.

[37] Fickel J, Wagener A, Ludwig A. Semen cryopreservation and the conservation of endangered species. Eur J Wildl Res 2007;53:81–9.

[38] Leibo SP, Songsasen N. Cryopreservation of gametes and embryos of non-domestic species. Theriogenology 2002;57:303–26.

[39] Zschokke S, Studer P, Baur B. Past and future breeding of the Indian rhinoceros in captivity. Int Zoo News 1998;45:261–77.

[40] Dennis PM, Rajala-Schultz PJ, Funk JA, Blumer ES, Miller RE, Wittum TE, et al. Risk factors associated with a skewed natal sex ratio in captive black rhinoceroses (*Diceros bicornis*) in the United States. J Zoo Wildl Med 2007;38:533–9.

[41] Linklater WL. Translocation reverses birth sex ratio bias depending on its timing during gestation: evidence for the action of two sex-allocation mechanisms. Reprod Fertil Dev 2007;19:831–9.

[42] Behr B, Rath D, Hildebrandt TB, Göritz F, Blottner S, Portas TJ et al. Index of sperm sex sortability in elephant and rhinoceros. Reprod Domest Anim, in press.