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EVALUATION OF FERTILITY IN CAPTIVE MALE WHITE RHINOCEROS *(CERATOTHERIUM SIMUM)* - SEMEN ASSESSMENT AND PRESERVATION

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Extended Abstract

Substantial knowledge on the reproductive physiology of the female white rhinoceros has been gathered over the last four years (for review: SCHWARZENBERGER et al., 1999). Driving force of these combined efforts was a low rate of reproduction of the captive southern white rhinoceros population. In female white rhinoceros a number of problems associated with this low rate of reproduction such as acyclicity and erratic cycles, mating failure, conception failure and pregnancy failure have been identified (San Diego Workshop, 1998) and suggestions to solve these problems have been made (ANONYMOUS, 1998, SCHWARZENBERGER et al., 1999). However, little emphasis has been put on the evaluation of male fertility as a possible contributing factor to the demographic crisis of the captive population considering that 81% of male white rhinos in the ESSP have not yet sired offspring (OCHS, 1999). Different methods of semen collection and basic physiologic data have been described for three rhinoceros species including data from one white rhinoceros (SCHAFFER et al., 1998a). In this study reproductive tract ultrasound, electroejaculation and manual semen collection were conducted in mature non-proven breeders to obtain further basic data on the reproductive physiology and the fertility status. Semen preservation and cryopreservation was performed as part of the development of assisted reproduction techniques in this species.

In 10 male white rhinos ultrasound of the genital was conducted in transrectal (accessory sex glands) and transcutaneous (testis and epidymis) manner. Two males were conditioned to tolerate manipulation and veterinary procedures in a restraint device (SCHAFFER et al., 1998b). In these two males penile stimulation techniques were performed one to three times per week over a period of one year. Manual penile stimulation, artificial vagina (40°C), penile hot towel compression (45-48°C) were applied at different times to induce an ejaculatory reflex. All other males were anaesthetised (GÖLTENBOTH et al., 2000; WALZER et al., 2000) for semen collection by electrostimulation (SCHAFFER et al., 1998a; HILDEBRANDT et al., 2000; HERMES et al., 2000). The semen collections were performed every second month throughout the year. Semen assessment was based on volume, sperm motility and morphological characteristics of the various fractions collected. The morphological integrity was evaluated after staining of smears as described for bull spermatozoa (BLOTTNER et al., 1989). For preservation and cryopreseravtion fractions were centrifuged and extended with egg yolk based DMSO buffer (BLOTTNER, unpublished data). Extended fractions were equilibrated for 2 hrs at 4 °C. Aliquots of the fractions were either kept at 4°C or cryopreserved in liquid nitrogen. Cooled samples were evaluated in regular intervals until motility had ceased. Post-thaw motility and morphology were evaluated. All data are given as means ± SEM. Electronmicroscopy was used to characterise the microstructure of native and cryopreserved spermatozoa.

The ultrasonographic imaging of the accessory sex glands (vesicular gland, prostate, bulbo-urethral gland), vas deferens, the epididymis and testis prior to the semen collection evaluated the dimensions

and present health status of the organs. Size, appearance and location of the prostate, vesicular gland and bulbo-urethral gland gave no indication for a potential organic disorder in any of the males examined. Imaging of the sex glands provided necessary information prior to electroejaculation, as this anatomical area represents the maximum point of sensitivity for stimulation. Ultrasound facilitated precise positioning of the later applied electrostimulation probe. Differences in size of the epidymis and testis were noted in individuals according to their age. Fibrosis of the testicular parenchyma characterised as hyperechoic spots was documented in males $\geq$ 19 years. The occurrence of multi-focal testicular fibrosis was higher in older males ($\geq$ 30a) compared to middle-aged males (10a $\geq \leq$ 30a) and just matured males ($\leq$ 10a) with no fibrotic spots detectable.

Routine manual penile stimulation resulted in the collection of 1-2 ml of ejaculatory fluid (n=20) in one of the two regular stimulated males. Arousal, complete erection and pulsatile contractions were achieved at each collection attempt after 10-15 min. of manual stimulation. However, a semen sample was collected in less than one out of four attempts. Mean sperm concentration was 0.262 x 10^9/ejaculate and 217.3 x 10^6 sperm/ml. The collected samples were macroscopically characterised by a clear fluid containing white, worm-like filaments. These filaments represented densely aggregated spermatozoa with 10-30% motility and no or little forward progression. Electron and light-microscopy showed the dense filamentary structure of the spermatozoa in these ejaculates. Immediate dilution with extender resulted in the dissolution of these filaments and an increase in motility by 40-50%. The diluted non-aggregated spermatozoa showed 70-80% motility with very good forward progression. However, post thaw motility of cryopreserved fractions did not exceed 10%. On two occasions only the ejaculatory volume increased to 4ml. These ejaculates consisted of a white uniform fluid, free of spermatozoa aggregates with a motility of 70-80% and good forward progression. Penile stimulation by an artificial vagina or hot towel compressions led to similar penile reflexes and contractions as when manually stimulated but did not result in the collection of ejaculatory fluids in either male.

Fractionated semen collection was obtained during 17 electrostimulation procedures in 8 male white rhinoceros. The ejaculate was collected in 3 – 12 fractions with a mean volume of 124 ± 21,1 ml. The mean number of spermatozoa per ml ejaculate and total number of spermatozoa per ejaculate was 48,11 ± 12,6 x 10^6 and 2.37 ± 0.8 x 10^9, respectively. A significant difference ($p \leq 0.1$) in the total number of sperm per ejaculate was noted when bulls $\geq$ 30 years (4.16 ± 2.3 x 10^9) were compared to those < 30 years (1.56 ± 0.36). Motility of cooled sperm decreased gradually to values of $\leq$ 30% after 4 days at 4°C. Post thaw motility of cryopreserved sperm varied between 30-50%. Filament like sperm aggregates as noted during manual stimulation were observed only during one electrostimulation and were associated with the smallest ejaculate volume obtained by electrostimulation (6.5 ml) and poor post thaw motility. Semen motility was consistently high ($\geq$ 60-95 %) in six bulls, inconsistent ($\geq$ 35 – 90 %) in two bulls and consistently non-sufficient ($\geq$ 10 %) in one bull. Urine contamination of the ejaculatory fractions as possible side effect occurred during 8 collections. The urine contamination occurred either at the end of a stimulation procedure due to over-stimulation (n=5) or in intermediate fractions due to misplacement of the electro-stimulation probe (n=3) and was characterised by an increase in volume and a decrease in motility of 25-40% and 45-65%, respectively in addition to changes in odour and colour. Slight correction of the electro-probe position in the case of a temporal probe misplacement resulted in the collection of contaminant free fractions.

Reproductive assessment provided accurate information on the breeding potential of male white rhinoceros with an implication on management decisions. According to the results of the reproductive assessments male white rhinos were rated as reliable (n=6), non-reliable (n=2) and non-sufficient (n=2) semen donors. Although all males examined were not represented in the captive population, in six bulls infertility due to spermatogenic insufficiency was excluded as a cause for missing reproductive success. Cause of the poor reproductive fitness in two males remained speculative. However, efforts to improve the male reproductive condition by translocation or introduction to new females as recommended by the EEP should be accompanied by reproductive assessments. In

general, electroejaculation resulted in the recovery of larger ejaculatory volumes when compared to manual stimulation and previously reported electroejaculation in the black rhinoceros (SCHAFFER et al., 1998). The use of a special designed hand-held stimulation probe provided better placement and more accurate stimulation of the sensitive pelvic area (HILDEBRANDT et al., 2000). Ultrasound was useful for the assessment of the health status and detection of potential disorders in the male rhinoceros reproductive organs. The testicular fibrosis first described in a rhinoceros was a common finding in mature males. However, it did not have a negative influence on the fertility parameters as older males showed high spermatozyte density and consistent high motility when repeatedly ejaculated. In bovine breeding bulls comparable testicular fibrosis has been observed starting at the age of four, 2.5 years after puberty. Histology showed that these hyperechoic spots corresponded with an increase of connective tissue in the testicular parenchyma and focal atresia of the germinal epithelial (BÜCHELER, 1994; GLATZEL, 1996; GRAUE et al., 2001). However, spermatogenesis or fertility parameters in bulls were not affected by the increasing occurrence of testicular fibrosis (GRAUE, unpublished data). SCHAFFER et al. (1998) discussed the manual stimulation and collection of ejaculatory fluid drops in a white rhinoceros as a result of a passive emission attributing the small volume to the unconditioned training status of the individual. Although two male white rhinos were conditioned over a long time on manual stimulation in this study the volume of ejaculatory fluids collected could not be increased to volumes similar to those recorded when electro-stimulated. Moreover, our observation of filament-like sperm aggregates suggest that spermatozytes stored in the vas deferens were passively emitted without further contribution of accessory gland fluids as reported by SCHAFFER et al. (1998). Sperm aggregates showed similar diameter as the sonographic and post mortem (HERMES, unpublished data) measures of the vas deferens in the rhinoceros. Although, motility was increased and aggregates dissolved by addition of semen extender, the deficiency of essential ejaculatory supplements was supposedly responsible for poor cryopreservation results. The collection of larger semen samples on two occasions was associated with mating behaviour and arousal prior to the manual stimulation procedure (MACLAUGHIN, pers. com.).

Preservation and cryopreservation have been performed as integral part of fertility assessments. Preliminary results of cryopreservation of epididymal sperm from hunted males with commercially available cryodiluents (equine diluent; Triladyl®)demonstrated the feasibility of cryopreservation in white rhinoceros (LUBBE et al., 1999). However, cryopreservation of larger sperm quantities, from greater numbers of males as part of fertility evaluations and basis for the development of assisted reproduction programs appears feasible only by means of electroejaculation unless further methods are developed to induce seminal fluid emission by manual stimulation.

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