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Artificial insemination in the anoestrous and the postpartum white rhinoceros using GnRH analogue to induce ovulation

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Abstract

The objective of this study was to develop AI and to achieve first time pregnancy in a nulliparous rhinoceros. For this, one 24-year-old irregular cycling female white rhinoceros was selected, which had never been mated. The endocrine function was monitored by faecal and serum pregnane analysis. Ultrasound determined the optimal day for AI by measuring follicle sizes of 2.0, 2.6, 3.0, 3.2 cm on days −6, −4, −1, 0 of the induced oestrous cycle, respectively. AI was performed and ovulation induced when a pre-ovulatory-sized follicle was present using GnRH analogue, deslorelin. Fresh semen was deposited in the uterine horn using a patented AI catheter overcoming the hymeneal membrane and torturous cervical folds non-surgically. Moreover, ultrasound monitoring of the uterine involution and ovarian activity on days 16, 26, 30 postpartum facilitated the induction of and the AI on the first postpartum oestrous in a rhinoceros using GnRH analogue. Two consecutive pregnancies were achieved by AI for the first time in the rhinoceros. Pregnancies were diagnosed by elevated serum and faecal 20-oxo-pregnane concentrations. In addition ultrasound measured biometric parameters of the two foetuses on days 86 and 133 of gestation. Two female calves were born after 490 and 502 days of gestation, yet one calf was stillborn. AI in rhinoceros might now be used as assisted reproduction technology tool to boost critically small captive rhinoceros populations.

Keywords: Reproduction; White rhinoceros; Artificial insemination; Ultrasound; Pregnancy

1. Introduction

Over the last few decades, rhinoceroses have become important icons in the saga of wildlife conservation. Recent surveys estimate the wild populations of the five rhinoceroses species, black (*Diceros bicornis*), white (*Ceratotherium simum*), Greater One-Horned (*Rhinoceros unicornis*), Javan (*Rhinoceros sondaicus*) and Sumatran rhinoceros (*Dicerorhinus sumatrensis*) to be, at most 3610, 11,330, 2400, 60 and 300 [1,2]. Successful reproduction of rhinoceroses in human care gained increasing importance with all species being critically endangered in the wild and distinct subspecies categorized by the ICUN as in ''imminent threat of extinction''. The northern white rhinoceros (*Ceratotherium simum cottoni*) and the eastern Sumatran rhinoceros (*Dicerorhinus sumatrensis harrissoni*),

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extinct in most of their natural habitat, remain with wild populations consisting of 4 and 13 individuals, respectively [3]. The western black rhinoceros (*Diceros bicornis longipes*) has recently been declared extinct by the IUCN [3]. This illustrates the dismal outlook for certain rhinoceros subspecies in the wild making successful ex situ breeding programs paramount for rhinoceros subspecies survival.

However, in captivity fundamental problems in the management of captive populations are the low reproductive rate in the white and the Sumatran rhinoceros and a male-biased offspring in the Greater One-Horned rhinoceros [4–9]. This limited breeding success in captivity greatly impairs efforts to maintain viable captive rhinoceros populations. For example in the captive southern white rhinoceros a rapidly ageing captive population supplementation of the captive population by new imports from the wild is widely practised [10,11]. However, in the captive northern white rhinoceros or Sumatran rhinoceros the overall survival of the entire species might depend on the successful population management in human care.

Specifically, in the white rhinoceros long non-reproductive periods have shown to have a detrimental impact on the genital health of nulliparous females resulting in age-correlated reproductive pathology, absent or erratic oestrous cycle activity, and conception and pregnancy failure [11,12]. Consequently, the reproductive life span of these nulliparous females is 10–20 years shorter compared to females, which had at least one offspring. Females, which had reproduced, are minimally affected by reproductive pathology, strongly suggesting that this asymmetric reproductive ageing process is prevented by pregnancy. Reports and anecdotal information from the Greater One-Horned and Sumatran rhinoceros on extensive reproductive pathology suggest that a similar asymmetric aging process also occurs in captivity and isolated wild individuals in the Asian rhinoceros species [6,13–15]. Therefore, the achievement of at least one initial pregnancy in young animals has been discussed as prophylaxis to sex steroid-dependent reproductive disorders in nulliparous rhinoceroses [11].

The reproductive biology of the white rhinoceros has been well documented [4,5,11,12,16–19]. Reports on reproductive anatomy, oestrous cycle length of 35 and 70 days, gestation length, ovarian dynamics in oestrous and anoestrous females, semen collection and preservation laid the foundation for the further development of assisted reproduction technologies. The objective of the present study was to develop an AI technique for the rhinoceros to achieve first time pregnancy in aged nulliparous females, to overcome the affects of long non-reproductive periods and to reduce currently long inter-calving intervals.

2. Materials and methods

2.1. Animals

One male and one female southern white rhinoceroses housed at the Budapest Zoo were selected for artificial insemination after multiple reproductive assessments [18,19]. The wild caught female (studbook # 902) and captive born male (studbook # 578) were housed together for 20 years. In the female, the intact hymeneal membrane upon clinical examination proved that the female had never been mated. Reproductive ultrasound examinations identified a small number of multiple endometrial cysts in the uterine body and horns, which did not exceed 0.2 mm. Since, in mares, limited number and size of endometrial cysts do not affect fertility, these lesions in the female rhinoceros were regarded as minor in respect to fertility, specifically when compared to more advanced stages of reproductive pathology in other non-reproductive female rhinoceroses [11,20]. Therefore, the female was considered as potential breeder. In the male, the previously assessed semen quality was satisfactory. In addition to the diet recommended by the husbandry guidelines both animals had been supplemented with 1.200 mg beta carotene per day starting 12 months prior to this study (Beta Karotin-Würfel, Salvana, Kl. O.-Sparrieshoop, Germany), because of its association with improved parameters of fertility [21,22].

2.2. Endocrinology

Endocrine function in the female was monitored by faecal progesterone metabolite analysis bi-weekly for over 6 years [4]. To compare faecal and plasma pregnane concentrations associated with oestrous and pregnancy, blood samples from the conditioned animal were taken from the ear vein once a week since January 2004. One additional plasma sample was collected 4 days after the first artificial insemination. During the postpartum period blood collection was hampered due to agitation. Therefore regular sampling started again 25 days after the second AI. Plasma and faecal samples were stored at −20 °C until analysis using a group-specific enzyme-immunoassay for 20-oxo-pregnanes [4,11]. This assay shows considerable cross-reactions with pregnane metabolites containing a 20-oxo-group and thus results of the plasma samples might, in

addition to progesterone, also include possible pregnane metabolites. Faeces were extracted as described previously [4]. For the extraction of plasma samples 0.2 ml of plasma were mixed with 5.0 ml of diethyl ether and vortexed for 30 min. Then the ether phase was transferred into a new vial, evaporated, and the residue was re-dissolved with assay buffer.

To induce and time ovulation in accordance with the AI, 4.2 mg synthetic GnRH analogue, deslorelin acetate (Ovuplant™, Peptech, Melbourne, Australia) were given at each AI attempt when ultrasound identified a pre-ovulatory follicle. AI-1 was performed in the nulliparous, anoestrous female, AI-2 was performed postpartum. The synthetic GnRH analogue was contained in a biocompatible sustained release subcutaneous implant specifically developed to improve the predictability of ovulation in cycling mares during the breeding season. In mares this synthetic GnRH analogue induced ovulation 24–48 h posttreatment by the increase in LH and FSH levels resulting from sustained release of the peptide [23–26]. The subcutaneous application of the two implants required a small surgical incision caudo-ventral to the ear that penetrated the rhinoceros dermis. Due to the extreme thickness of the dermis in rhinoceros and associated difficulties to access the subcutis the applied implants were not removed.

2.3. Anaesthesia

Both AIs (1/2) required one or multiple assessment of the ovarian status prior to insemination in the standing sedated female using 1.1 ± 0.3 mg/animal Ethorphine, 5 ± 1.0 mg/animal Acepromazine (Large Animal Immobilon® C-Vet Veterinary Products, Lancs, UK), 15 ± 2.0 mg/animal Detomidine (Domosedan®, Orion Corporation, Farmos, Finland) and 15 ± 2.0 mg/animal Butorphanol (Torbugesic®, Fort Dodge Animal Health, IA). All anaesthetics were injected into the neck muscles caudo-ventral to the ear using a dart pistol. Two minutes after the partial reversal with 50 mg/animal Naltrexone (Trexonil®, Wildlife Laboratories Inc., Fort Collins, CO) IV into the ear vein the female was alert and walking [11].

Artificial insemination and electroejaculation required a full anaesthesia using 3.1 ± 0.6 mg/animal Ethorphine, 12.5 ± 2.5 mg/animal Acepromazine, 12.0 ± 2.0 mg/animal Detomidine and 12.0 ± 2.0 mg/animal Butorphanol. An additional IV injection of ketamine at a dose of 150 ± 50 mg (Narketan®, Chassot AG, Bern, Switzerland) injected into the ear vein reduced the time to lateral recumbence. Recumbent animals received supplemental oxygen at a rate of 15 l/min through a nasal tube. For reversal 250 mg/animal Naltrexone and 20 mg/animal Atipamezole (Antisedan®, Orion Corporation, Farmos, Finland) were administered IV [27]. In the female local anaesthetic, lidocaine-hydrochloride Gel (Xylocain® Gel 2%, AstraZeneca GmbH, 22876 Wedel, Germany) was applied to the slightly parted vaginal labia to avoid reflex reactions when manipulating the clitoris during insemination.

2.4. Reproductive ultrasound and semen collection prior to artificial insemination

To determine the reproductive and the ovarian status the female genital organs were evaluated by ultrasound approximately 1 week prior to an expected behavioural unrest and increased male interest. To visualise the ovaries an ultrasound probe extension was required (SonoSite 180 Plus, C60 5-2 MHz probe, Product Group International Inc., Lyons, CO 80540) [11]. Pregnancy diagnosis and foetal development were evaluated for AI-1 on day 86 and AI-2 on day 133 of gestation in the standing sedated animal using 3-dimensional (3-D) ultrasound system (Voluson 730, GE Medical Systems, Austria). In the postpartum female ultrasound examinations were conducted on days 16, 26 and 30 following parturition to determine a possible postpartum oestrous. All ultrasound examinations were recorded on DV tape for retrospective analysis. Foetal measurements of different biometric parameters were taken: crown-rump-length (CRL), biparietal diameter (BPD), thoracic diameter (TH) and humerus, radius and ulna length (HL, RL, UL). Biometric measurements from the foetus were extracted directly from on board stored scans and processed with 4D View software (GE Medical Systems, Austria).

Semen for AI-1 and AI-2 was collected by means of electroejaculation (Seager model 14, Dalzell USA Medical Systems, The Plains, VA, USA). The semen samples were immediately diluted (1:1) with pre-warmed (37 °C) egg-yolk extender (buffer solution containing 2.41% (w/v) TES, 0.58% (w/v) Tris, 0.1% (w/v) fructose and 5.5% (w/v) lactose supplemented by 20% egg yolk (final concentration 15.6% (v/v)), and 20 IU (tocopherol/ml), maintained at room temperature (23–25 °C) and then 45–60 min after collection warmed to 37 °C prior to insemination). Ejaculate volume, spermatozoal concentration, total sperm number, sperm motility and morphology were assessed as previously reported for the rhinoceros [19,18]. For both AIs whole ejaculates were inseminated.

2.5. Artificial insemination

A single insemination was performed per induced oestrous cycle in the anaesthetized female with sperm collected approximately 1 h prior to the procedure. For the insemination a specific catheter was developed (Patent: DE 10203094A1, Chirurgiemechanik Schnorrenberg, 15569 Woltersdorf, Germany). The 115 cm long catheter was composed on the outside of a conic (4–9 mm), flexible carbon sheath with a handle. On the inside the carbon sheath held a 90 cm luer-lock cannula, which ended in an angled, smoothly edged stainless steel catheter tip. The catheter was designed to overcome non-surgically the hymenal membrane and the extremely tight cervical folds [16], the two main anatomical obstacles for intra-uterine insemination in rhinoceros.

3. Results

Two consecutive pregnancies were achieved by artificial insemination for the first time in a rhinoceros. For the first artificial insemination in the female displaying irregular oestrous cycle pattern the development of a dominant follicle to a pre-ovulatory-sized follicle was monitored on days −8, −1 and 0 of the induced oestrous cycle. For the second insemination uterine involution and ovarian activity were assessed on days 16, 26 and 30 postpartum or days −14, −4 and 0 of the induced postpartum oestrous, respectively. When a pre-ovulatory-sized follicle was present, ovulation was induced using a GnRH analogue on the day of insemination.

3.1. Timing of artificial insemination and oestrous induction

Hormone analysis classified the female as having irregular luteal activity throughout a monitoring period of 33 months prior to AI (Fig. 1). Despite the absence of regular pregnane elevations the male showed regular increased interest in the female. Therefore it was assumed that the female developed pre-ovulatory follicles without ovulating. One week prior to a period of increased male interest, on day −6 of the induced ovulation ultrasound determined the presence of a 2.0 cm follicle among several smaller follicles on the right ovary. On days −1 and 0 of the induced ovulation the diameter of dominant follicle had increased to 3.0 and 3.2 cm, respectively (Fig. 2A–C). The endometrial diameter at the *bifurcatio uteri* on days −6, −1, 0 of the oestrous cycle had increased from 2.2, 3.2 to 3.8 cm,

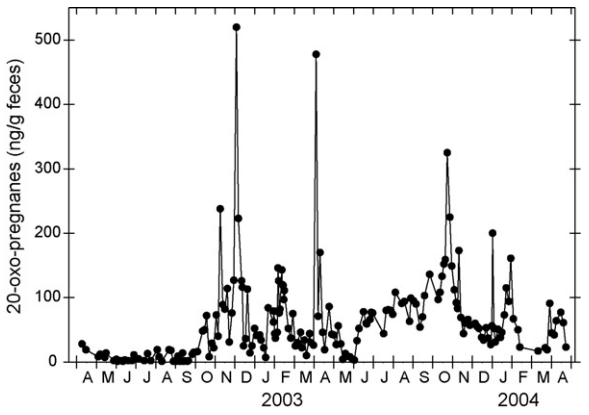

Fig. 1. Two year faecal 20-oxo-pregnane concentrations in a female white rhinoceros displaying irregular luteal activity.

respectively. The endometrial height of 3.8 cm and follicle diameter of 3.2 cm were considered as Graafian follicle, thus artificial insemination was performed and GnRH analogue given to induce ovulation shortly after the insemination.

Timing of the second AI was based on the assessment of uterine involution and the onset of follicular development postpartum. On day 16 postpartum the uterine horn which previously carried the foetus contained large amounts of fluid. The opposite uterine horn was free of fluid with an endometrial diameter of 2.0 cm. Follicular activity on the ovaries was limited to the presence of small follicles ≤1.0 cm. On day 26 postpartum only small amounts of fluid remained in former foetal carrying uterine horn. The endometrium had increased to 3.2 cm and a dominant follicle of 2.6 cm had developed. On day 30 postpartum a pre-ovulatory-sized, pear-shaped follicle of 3.2 cm was detected accompanied by an endometrial diameter of 36 mm at the bifurcatio uteri and a minimal amount of free fluid in the previously foetal carrying uterine horn (Fig. 2D–F). GnRH analogue was given on the day of insemination. The follicle growth rate in both induced oestrous cycles was calculated at ∼0.2 mm/day.

3.2. Insemination

The sterile insemination catheter was first guided into the vagina by digital palpation of the vaginal opening in the hymeneal membrane. The cervix was then palpated transrectally and manually fixed while the catheter was manoeuvred through the cervical folds and the catheter tip positioned in the uterine horn on the side the pre-ovulatory follicle was present. Transrectal ultrasound verified the correct placement of the catheter in the uterine horn. The deposition of the pre-warmed

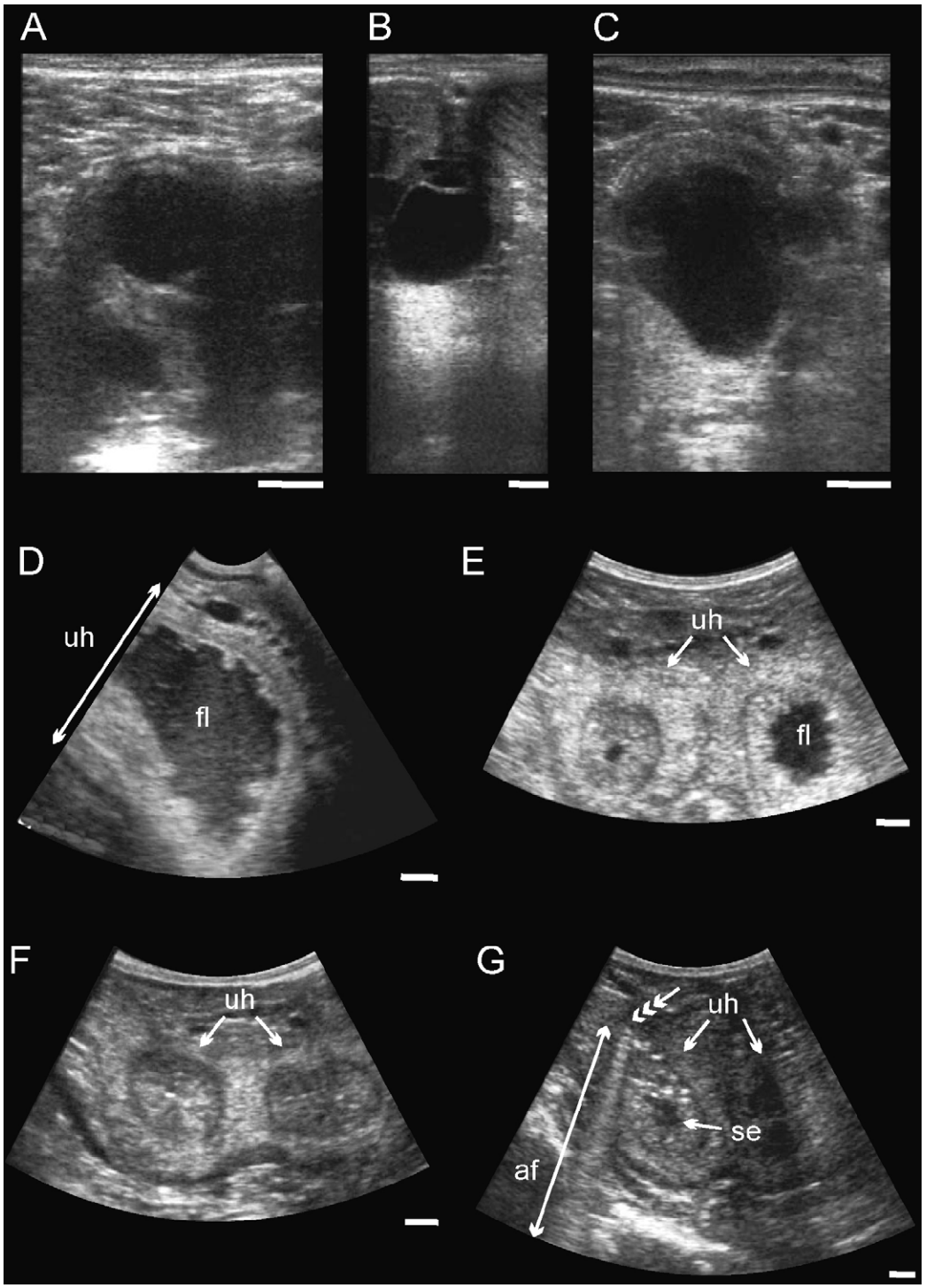

Fig. 2. (A–C) Sonograms of the dominant follicle (Ø 22, 32 and 38 mm) in a white rhinoceros on days −6, −1 and 0 of the induced oestrous cycle. Note the pear-shaped structure of the Graafian follicle. (D–F) Uterine involution postpartum: sonograms of the uterine horns in cross-section on days 16, 26 and 30 p.p. (D) Day 16 p.p.: uterine horn (uh) was enlarged and contained large volumes of fluid (fl). (E) Day 26 p.p.: the previously foetal carrying right uterine horn (uh) contained small amounts of fluid (fl). The contra lateral horn, the side on which the next dominant follicle developed, was involuted and free of fluids. (F) Day 30 p.p.: uterine involution and fluid resorption was completed in both horns (uh). (G) AI 30 p.p.: verification of catheter position during AI. Catheter was placed in the uterine horn (uh) on which ovulation was going to occur. The metal tip of the AI catheter was visible as dense echogenic dot in cross-section (←) creating a shadow artefact (af ↔). Semen (se) was visible as low echogenic fluid in the uterine lumen.

Table 1
Semen characteristics of a white rhinoceros male, donor for AI-1 and AI-2

Artificial insemination (date)	Volume (ml)	Sperm concentration ($\times 10^6$/ml)	Total motility (%)	Progressive motility (0–5)	Sperm with normal morphology (%)
08 April 2004	88	120	80	4	50.5
08 September 2005	58	100	90	4	19

(37 °C) and extended (1:1, v/v) semen was monitored by ultrasound (Table 1 and Fig. 2G).

3.3. Pregnancy monitoring and parturition

Serum 20-oxo-pregnane concentrations started to increase around day 4 following GnRH analogue treatment indicating that ovulation had been successfully induced, timed with the artificial insemination. In contrast to the rise of serum pregnane concentrations within the first week following ovulation, faecal 20-oxo-pregnane concentrations were more variable and indicated corpus luteum development only around 3 weeks after the GnRH application, documenting a considerable delay of progesterone metabolites occurrence in the faeces. Serum pregnane concentrations remained elevated throughout the pregnancies until shortly before parturition (0.8–40 ng/ml plasma) with a steep increase of concentrations above 6 ng/ml plasma occurring around days 180 and 110, during the first and the second pregnancy, respectively. In contrast to the elevated serum pregnane concentrations from start to end, faecal 20-oxo-pregnane concentrations were less conclusive during the first 2.5 months of pregnancy. Thereafter faecal pregnane concentrations steadily increased remained at high levels throughout pregnancy. One day prior to parturition serum plasma pregnane concentrations dropped markedly by about 80% from 40 to 7 ng/ml plasma as a clear indicator for the imminent onset of labour after 490 and 502 days of pregnancy, respectively (Fig. 3).

Three-dimensional ultrasound and Colour Doppler ultrasonography verified foetal integrity, heart activity, umbilical cord blood flow and biometric parameters, thus the presence of a viable foetus on day 86 (AI-2) and on day 133 of gestation (AI-1) (Table 2 and Fig. 4). The implantation site in the uterine horn coincided for both pregnancies with the ovary on which ovulation had occurred. After full term pregnancies two female calves were born on day 490 (AI-1) and 502 (AI-2) of gestation. While the second calf from AI-2 was live the first calf had been stillborn. Premature placenta detachment evident by large amounts of bloody discharge early during the labour process caused presumably foetal cardiac arrest. The calf could not be revived when delivered 7 h after placental detachment.

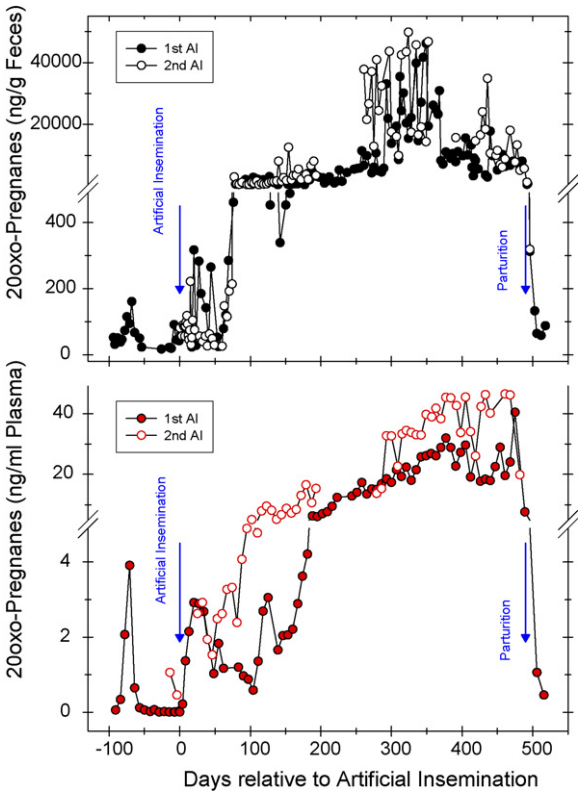

Fig. 3. Comparative faecal and plasma 20-oxo-pregnane concentrations during two consecutive pregnancies from AI in one white rhinoceros.

Table 2
Biometric measurements from two white rhinoceros foetus after AI-2 and AI-1 on days 86 and 133 of gestation, respectively

Gestation (days)	Pregnancy from AI	CRL (mm)	TH (mm)	BPD (mm)	RL (mm)	UL (mm)
86	2	82	29	25	–	–
133	1	177	70	44	15	20

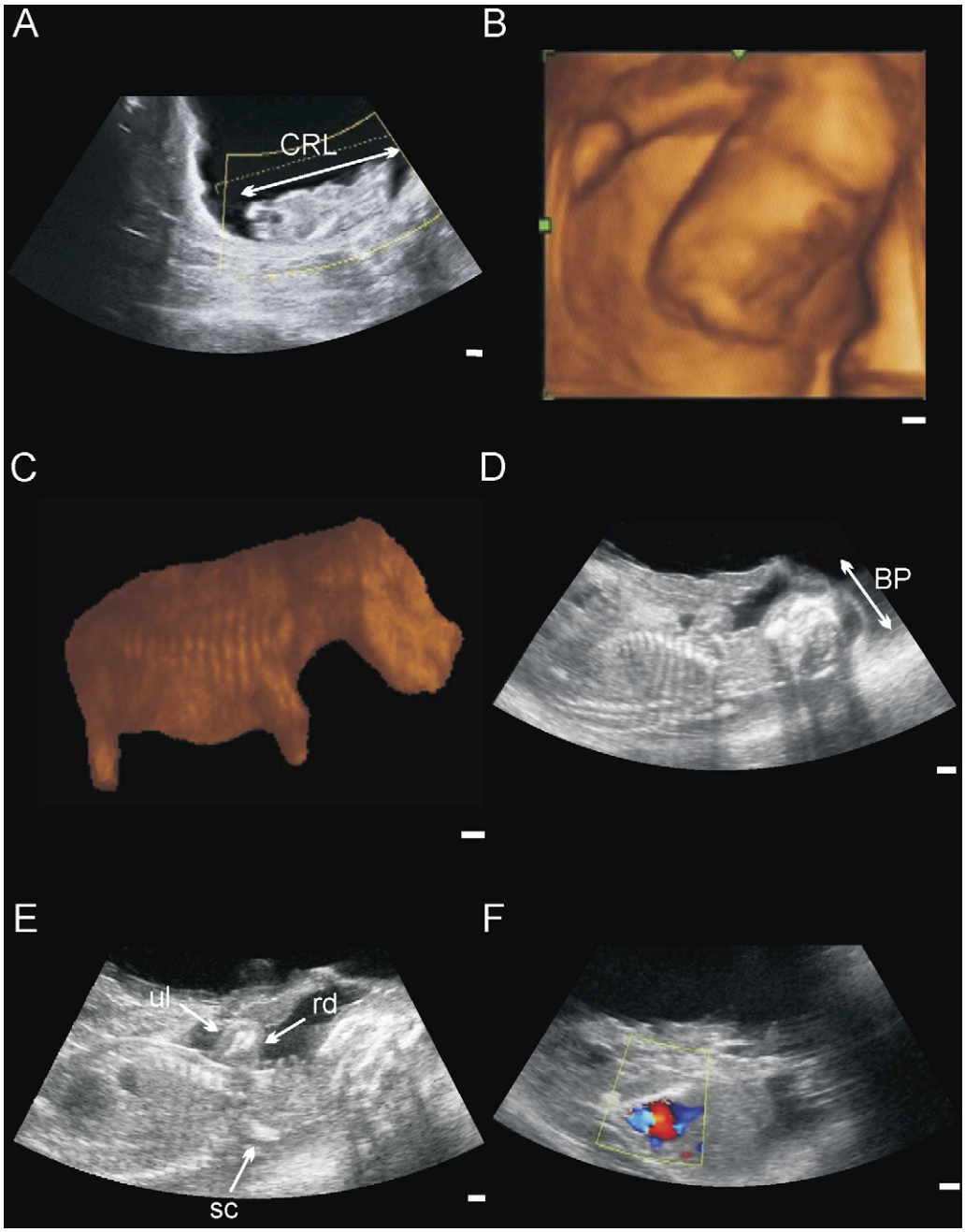

Fig. 4. 2-D and 3-D sonograms of white rhinoceros foetuses. (A/B) Eighty-six days of gestation post AI-2. (A) 2-D crown to rump view (CRL) of the foetus. The region of interest (yellow) for 3-D scanning was chosen at maximum CRL. (B) 3-D sonogram of the complete foetus within the foetal cavity: head, thorax, abdomen, front and hind legs were now clearly distinguished. (C–F) One hundred and thirty-three days of gestation post AI-1: (C) 3-D sonogram of the complete foetus with rhino specific features. Note the horn plate on the rostrum. (D/E) 2-D images of the foetus from the 3-D data set: (D) head with the biparietal distance (BP). (E) Thorax (th), radius (rd), ulna (ul), scapula (sc). (F) Colour Doppler image of the foetal heart. Heart ventricle (red) and atrium (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

4. Discussion

This is the first report of successful AI in the rhinoceros. Two consecutive pregnancies have been achieved by AI in a 24-year-old and previously nulliparous and irregular cycling white rhinoceros. Specific achievements of this study were the monitoring of follicular growth and ovulation induction using

GnRH analogue on the day of AI, the intra-uterine insemination through the long, tight and tortuous rhinoceros cervix and the second AI with subsequent pregnancy on the postpartum oestrous.

The integration of AI as assisted reproduction technique to enhance captive rhinoceros breeding programs has been discussed and desired for over 15 years [4,5,6,16,28]. When considering the limited breeding success of both white rhinoceros subspecies in captivity, skewed birth sex ratios in the Greater One-Horned and black rhinoceros [6,7], and the disastrous situation of some rhinoceros subspecies in the wild [3,10,29] advanced assisted reproduction seem greatly underutilized to overcome these crisis. The role of assisted reproduction technologies has recently been acknowledged as increasingly important to the success of captive breeding management, specifically in those species with a dismal outlook of survival both in captivity and in the wild [6,29]. Intensive use of assisted reproduction technologies to increase reproductive rate in captivity now becomes almost mandatory. Hence, artificial insemination, as now demonstrated in the rhinoceros, provides reproductive physiologists and conservationist with a potent instrument to re-establish viable, self-sustainable captive populations of imminently threatened rhinoceros species and to maximize the genetic diversity when using unrepresented captive or wild semen donors [10,19].

Accurate timing of ovulation is most crucial for the success of an artificial insemination. The ultrasound monitoring of the developing dominant follicle combined with ovulation induction on the day of insemination using GnRH analogue facilitated optimal timing. Follicular development has been described in different rhinoceros species showing a great variance in the size of the Graafian follicle. Ovulatory follicles in the black, white, Greater One-Horned and Sumatran rhinoceros are 4.9, 2.8–3.2, 10.0–12.0 and 2.0–2.5 cm, respectively [6]. The follicular growth rate in the black rhinoceros of ∼0.3 cm/day in ovulatory follicles with sizes of ∼5.0 cm [39] is in comparison with the calculated follicular growth rate in the white rhinoceros of ∼0.2 cm/day in this study. However, in all four rhinoceros species in captivity regular follicular waves and pre-ovulatory follicles may not summit in ovulation but in the formation of haemorrhagic or artretic, anovulatory follicles [6,28,38–40]. Specifically in the white rhinoceros a large number of young females with active ovaries and follicular development fail to ovulate and to start normal oestrous cycle pattern [11,12]. The formation of anovulatory follicles in the rhinoceros has been discussed as being similar to ovarian events during the transition period form non-breeding to the breeding season in the mare [28,39–41]. The objective in this study was to induce ovulation close to the insemination in a non-cycling female. Two hormone treatments have been published, which induced the oestrous cycle of white rhinoceroses. Both of these hormone treatments, based on long term down regulation of the ovarian activity using synthetic progesterone + hCG or long acting GnRH + hCG, were of limited success: few animals responded, timing of ovulation was inaccurate and the goal to induce a normal oestrous cycle pattern was not achieved [4,11].

GnRH agonist implants, deslorelin acetate, have been routinely used to hasten ovulation in mares during the breeding season as well as in transitional mares [23,24]. Therefore, this short-term GnRH agonist (Ovuplant^{TM}) was employed for the first time in the exotic species of the perissodactyla (wild equids, tapirs and rhinoceroses) to induce ovulation in a non-cycling female. When administered to mares with a pre-ovulatory-sized follicle the GnRH agonist hastened ovulation within 2 days in over 88% of cycling mares [23,25]. GnRH agonist also hastened ovulation during the first postpartum oestrous in the mares achieving normal fertility [42,43]. In the white rhinoceros, similar to transitional mares, GnRH short-term agonist induced ovulation after AI when a pre-ovulatory follicle was present in the anoestrous and the postpartum female documented by increasing pregnane concentrations and conception following GnRH treatment. However, when using GnRH agonist implants in mares, a delayed return to oestrus has been reported when mares failed to become pregnant due to prolonged absorption of deslorelin [44,45]. The removal of subcutaneous GnRH implants after ovulation resulted in normal inter-oestrous intervals [43,46]. Since such removal of deslorelin implants at a time ovulation has occurred is not feasible in rhinoceros due to the extremely think dermis and the necessary anaesthesia we suggest the use of injectable short-term release deslorelin to circumvent the potential prolongation of interovulatory interval in the rhinoceros when conception fails [43,46,47]. Serum progesterone results indicated that GnRH agonist treatment induced ovulation in the white rhinoceros within 48 h when pre-ovulatory sized follicle was present similar to the mare [48]. This is of important relevance to the large number of anoestrous females with ovarian activity in the captive population. Serial ultrasound characterization of pre-ovulatory follicles in anoestrous females combined with an efficient hormone treatment could be broadly used for either natural or assisted breeding attempts. However, further investiga-

tions and larger study sizes are necessary to evaluate the efficiency of GnRH agonist, deslorelin to induce ovulation reliably in rhinoceros species.

Anatomical challenges for the AI in the rhinoceros were the hymeneal membrane in nulliparous females which have never mated and, more difficult, the firm cervix with its extreme tortuous cervical canal between large folds of dense fibrous connective tissue [16]. The hymeneal membrane, present in 76% of captive nulliparous female white rhinoceros [11], was located by digital palpation and the AI catheter tip was then passed into the vagina. The passage of the cervix required the specific catheter design. To overcome right angle turns and blind pockets during the passage through the 15–20 cm cervix the AI catheter material had to endure 90° flexibility, when at the same time the catheter tip had to be smooth-round edged to avoid tissue perforation. Permanent forward pressure and constant turns of the 45–60° flexed catheter allowed the non-surgical, trans-cervical approach to the uterus. Further it facilitated the precise intra-uterine semen placement in the uterine horn on which ovulation occurred. This non-surgical access to the uterus in a rhinoceros has potential applications other then AI such as uterine biopsy, drain of intra-uterine fluid accumulation [11,55]. Deep intra-uterine low dose inseminations using sex-sorted sperm or embryo transfer after IVF are further advanced reproduction technologies in the future, which can be performed in the rhinoceros with this non-surgical, trans-cervical uterine approach.

Pregnancy was diagnosed by elevated pregnane concentrations and ultrasound [4,5,38]. The comparative measurement of plasma and faecal pregnane concentrations demonstrated the delay of pregnane concentrations in the faeces as compared to plasma immediately after ovulation. When plasma pregnane concentrations rose 4 days after GnRH treatment, faecal pregnane concentrations remained low until indicating luteal activity only 2–4 weeks after oestrous induction. A steep increase of faecal pregnane concentration ∼70 days after ovulation indicated pregnancy [4,5]. This second pregnane increase was not so profound in the plasma as compared to faeces. The plasma pregnane concentrations described events following ovulation more precisely, but for accurate information on the pregnancy status in the white rhinoceros faecal pregnane provided earlier and more reliable information 3–4 months postconception

The embryonic vesicle can be detected as early as 15 days postovulation in the white rhinoceros [38]. Due to the lack of a restraint chute in most facilities, sedation is required for a pregnancy ultrasound examination. However, if females fail to become pregnant, the sedation poses an unnecessary intervention. Therefore pregnancy ultrasounds in this study were performed when pregnane concentrations remained elevated ∼70 days after conception visualizing the foetuses in an advanced state of the first trimester. The use of 3-D ultrasound facilitated access to detailed foetal biometric data, which otherwise is not accessible in a short, time constraint 2-D ultrasound examination under sedation. Biometric parameters such as the crown-to-rump-length, biparietal diameter, thorax width, humerus, radius and ulna length were documented during the examination. The 3-D ultrasound data set allowed the retrospective virtual turn of the foetus in three axes facilitating the optimal view and length of these developmental hallmarks and their exact measurement. Long term monitoring of foetal growth and relating 3-D ultrasound measurements to the foetal age would allow the establishment of accurate foetal growth curves for the rhinoceros with multiple anatomical features as demonstrated in the elephant, a species with comparable body and foetal dimensions to the rhinoceros [56]. In preparation for parturition and to avoid maternal aggression with the consequence of possible infant death, the accurate estimation of gestational length and prediction of parturition is of outmost importance to animal managers. However, in open range management systems in which animals are left outside overnight, mating might occur unnoticed and conception date remains unknown, thus gestational length is difficult to determine. Therefore, accurate foetal growth curves would provide fundamental physiological and management data to determine the gestational length even if females were examined and foetal measurements were taken only once.

Nulliparous female rhinoceroses with long, non-reproductive periods suffer from a multifaceted spectrum of pathological lesions in the genital tract, defined as asymmetric reproductive aging [11,12]. This asymmetric reproductive ageing is regarded as a continuous process occurring in nulliparous females, first evident around 15 years of age, which leads to infertility and premature senescence 10–15 years earlier compared to reproducing females in captivity or in the wild. This non-reversible process has important consequences for the management and survival of captive rhinoceros populations, as assumed reproductive life spans are immensely shortened. Substantial evidence among several species including the rhinoceros suggested pregnancy to prevent genital tract lesions occurring from this reproductive, hormone-dependent syndrome, thus preserving fertility [11]. Therefore, we

suggest to implement artificial insemination as management tool to achieve first time pregnancies in young and/or non-reproducing females to obtain prophylaxis to asymmetric ageing, to maintain and to preserve female genital health. Since extensive reproductive disorders (tumours, ovarian cysts) in non-reproducing females inflict permanent pain and discomfort early pregnancy by AI can be viewed as an animal welfare requirement.

This study reports on a pregnancy after first postpartum oestrous in a rhinoceros. Ultrasound documented the postpartum involution of the uterus, complete resorption of intra-uterine fluid accumulation and the development of a pre-ovulatory follicle by 30 days postpartum. In the wild, intervals between births in white rhinoceroses range from 2.63 to 3.45 years [49]. However, single reports document possible shorter intervals between successive births of 17, 18 and 21.5 months suggesting the existence of a postpartum oestrous in the white rhinoceros [50–52]. Based on endocrine data postpartum oestrus has been suggested in the black rhinoceros [53] and the manipulation of the postpartum period by means of management to reduce inter-calving intervals and to increase the reproductive rate of rhinoceroses have been discussed [49]. The prediction of oestrous is one of the major difficulties in the application of artificial insemination in the white rhinoceros. The data presented on the 30 days puerperium until first inducible postpartum ovulation facilitates managers with the ability of accurate timing for assisted breeding purposes in postpartum rhinoceroses. In mares, fertility during first postpartum oestrous is equally high to pregnancy rates in subsequent cycles [54]. The intensive use of AI at postpartum oestrous in rhinoceroses could further substantially increase captive reproductive rates.

With the current reproductive rate and population size of the five potential breeders, the northern white rhinoceros population in human care is doomed for extinction. In 57 years of captive management, only four captive births have been recorded and in the past 22 years, only one birth occurred [10]. The calculated survival rate in 50 years is less than 1%. In contrast to conventional breeding management, the use of AI might represent a new tool with immense positive influence on the population demography and population survival in captivity.

To further increase critically small populations to a self-sustainable population size in a very short time, a higher number of female offspring would be desirable. Specifically in a species with a long inter-calving interval of 1.5–3.45 years [49–52], the number of females available for breeding limits the rate at which a population can grow. In domestic species and few wildlife species the use of sex sorted sperm is described as a new tool to influence the sex of the offspring by using sex-biased sperm samples [30–37]. AI in rhinoceros now facilitates the development and implementation of sex-sorted spermatozoa to boost critically small captive rhinoceros populations by producing predominantly female offspring using X-chromosome bearing spermatozoa. We therefore conclude, that AI might evolve as an extremely valuable assisted reproduction technology in the future captive management of rhinoceroses.

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