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Theriogenology xx (2012) xxx

Theriogenology

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Estrus induction in white rhinoceros (*Ceratotherium simum*)

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Received 27 January 2012; received in revised form 14 May 2012; accepted 22 May 2012

Abstract

The estrous cycle length in the white rhinoceros (*Ceratotherium simum*) is either 4 or 10 wk. The cause(s) for this variation as well as the poor fertility rate in captivity remains under debate in this species. Most captive adult white rhinoceros undergo long anovulatory periods without luteal activity which are considered a major reason for their low reproductive rate. In this study, the synthetic progestin chlormadinone acetate (CMA) was tested in combination with hCG or the GnRH analogue deslorelin for its efficiency to induce ovulation in fourteen females without luteal activity and in three, regular cycling females. HCG (N = 12), injectable GnRH analogue (N = 8) and GnRH analogue implants (N = 15) were given to induce ovulation after CMA treatment. Treatment success was determined using both transrectal ultrasonography and progesterone metabolite EIA analysis. A preovulatory sized follicle (3.5 ± 0.1 cm) or a corpus luteum (5.1 ± 0.7) was present on the ovary one day after induction in 93.1% of the treatments. Despite this high rate of ovarian response, ovulation rate differed between the study groups. The ovulation rate for hCG, injectable GnRH analogue and GnRH analogue implants was 66.7%, 62.5% and 93.3%, respectively. Ovulation rate in cyclic females treated with GnRH implants was 100% (6/6) compared with 89% (8/9) in females without luteal activity receiving the same treatment. The length of the estrous cycle when induced with hCG was 4 wk (85.7%). The estrous cycle when induced with GnRH analogue was predominantly 10 wk long. Two females without luteal activity treated with GnRH became pregnant. In conclusion, CMA in combination with GnRH analogue implants was highly effective to induce ovulation in white rhinoceroses and thus can contribute to efforts aimed at increasing natural mating and reproductive rates in the captive white rhinoceros population.

Keywords: Assisted reproduction; Anestrous; Ultrasound; Chlormadinone acetate; GnRH

1. Introduction

Based on non-invasive fecal steroid monitoring, two types of estrous cycles of approximately either 4 or 10 wk in length have been described for the white rhinoceros (*Ceratotherium simum*) [1,2]. Due to low reproductive rate in captivity, the correlation between estrous cycle length and fertility is not yet clear. One previous study noted that a 10-wk cycle related to embryo resorption [3]. Other studies determined that the majority of captive females (61%) displayed long anovulatory periods which were only occasionally interrupted by erratic luteal activity [1,2]. These anovulatory periods are therefore considered a primary

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0093-691X/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2012.05.015

reason for the low reproductive rate in captivity for this species.

Historically, 74% of all wild-caught white rhinoceroses (N = 199/269) and 87% of all the captive born animals (N = 239/274) have never reproduced. More specifically, only 26% of all wild-caught females and 18% of captive born females ever reproduced in captivity. Recently imported animals have not reproduced at better rates, leaving the European captive population far from self-sustaining [5]. Reasons for this low reproductive rate seem multifold. Studies on the luteal activity in white rhinoceroses reported that 61% of all captive females were anovulatory without luteal activity. Specifically in young, mature females this finding was of great concern [1,2,6]. Ultrasonography of the reproductive tract in aged females, >15 yr of age, revealed that long non-reproductive periods were associated with extensive pathologies limiting or even excluding successful reproduction [4,6]. No such pathologies were found in young females without luteal activity which were presumed to be reproductively healthy. Yet reasons for their permanent anovulatory status were not determined [6]. The alarming endocrine and sonographic data strongly suggested that the lack of luteal activity thus the lack of regular ovulation represents a major impediment to a successful captive breeding program.

Several protocols for hormonal induction of ovulation have been attempted in different rhinoceros species [7]. Protocols using synthetic progestin, sometimes preceded by $PGF_{2\alpha}$ and followed by different combinations of FSH, PMSG, hCG, and GnRH have largely failed to reliably induce ovulation. So far, only two protocols have been reported to synchronize estrus in anovulatory white rhinoceroses: (1) synthetic progestin, chlormadinone acetate combined with hCG, and (2) GnRH analogue, deslorelin acetate applied when a preovulatory sized follicle was present without preceding synthetic progestin treatment [1,6,8,9]. The 45-day synthetic progestin treatment combined with hCG achieved an ovulation rate of 30% [6]. Induction of estrous using just GnRH analogue required close ultrasound monitoring of follicular development and was therefore limited to one individual.

The aim of the present study was to further test the efficiency of a synthetic progestin, chlormadinone acetate, when combined with either hCG or with a GnRH analogue in order to synchronize estrus and initiate spontaneous estrous cycles in anovulatory white rhinoceroses.

2. Materials and methods

2.1. Estrus induction

Fourteen females without luteal activity and three females with regular luteal activity were selected and some of those repeatedly treated for estrus and ovulation induction (Table 1). In animals selected for this study fecal pregnane had been monitored for 6 mo to several years. A total of 35 estrus induction treatments were conducted. All rhinos studied were registered in the international studbook for the white rhinoceros. For estrous induction oral synthetic progestin, chlormadinone acetate (30 mg/day) was given for 45 days (Synchrosyn, Pfizer, Berlin, Germany or Synchrosyn Alverta u. Werfft, GmbH, Vienna, Austria). The 45 day CMA treatment was designed to last longer than the calculated physiological life time of a corpus luteum. If a corpus luteum had just formed prior to the start of the treatment, synthetic progesterone mimicked luteal activity longer than the CL's life time, until its withdrawal initialized the development of a new dominant follicle. In cycling females this design allowed starting the CMA treatment even if the exact stage of the estrous cycle was not known.

On day 9.5 ± 0.2 upon completion of the synthetic progestin treatment, either hCG or one of two different formulations of GnRH analogue Deslorelin were applied. Seven anovulatory females, were treated with 10 000 IU of hCG (Ovogest, Intervet, Unterschleiβheim, Germany). Average time between the repeated use of hCG in the same female was 18 mo with a range of 7 to 46 mo. Six anovulatory females were treated by intramuscular injections of 3.0 mg of oily suspended GnRH analogue deslorelin acetate (BioRelease deslorelin, BET Pharm, Lexington, KY, USA). Further nine anovulatory and six ovulating, regular cycling females were treated with 4.2 mg of the GnRH analogue deslorelin acetate, suspended in two sustained release implants (Ovuplant, Selectavet Dr. Otto Fischer GmbH, Weyarn/Holzolling, Germany). Both GnRH formulations, the BioRelease oil and the sustained release implants, were designed to mimic the pulsatile nature of endogenous GnRH by releasing the GnRH analogue over a period of 48 h. Females received the Deslorelin by either hand injection or remote dart delivery [10]. Remote delivery of Deslorelin was achieved by standard 3 mL blow dart or specific implant-dart (Transponder blow dart TS-ID 100, Telinject, Römerberg, Germany). Ultrasound (Voluson i, GE Healthcare, Germany, with 2–5 MHz and 4–8 MHz transducers) assessed the ovarian status 1 day after GnRH analogue application in 18 treated females [6,9,11].

Table 1
Estrous synchronization in anestrous and estrous white rhinoceroses using chlormadinone acetate combined with either hCG or GnRH analogue.

Location	Studbook number	Age (years)	Group size (male.female)	Estrous (prior treatment)	Last CMA treatment	Ovulation trigger	Dominant follicle (cm)	Luteal activity (days)
Salzburg, AT	361	23	2.2	Anestrous	15.07.1994	hCG	n.r.	30
Salzburg, AT	361	24	2.2	Anestrous	01.05.1995	hCG	n.r.	30
Salzburg, AT	361	28	2.2	Anestrous	01.03.1999	hCG	n.r.	70
Salzburg, AT	361	28	2.2	Anestrous	13.12.1999	hCG	3.2	30
Salzburg, AT	361	30	2.2	Anestrous	11.07.2000	hCG	3.2	No luteal activity
Salzburg, AT	361	29	2.2	Anestrous	25.2.2001	hCG	3.2	30
Schwerin, DE	403	29	1.1	Anestrous	18.10.1999	hCG	CL 6.0	30
Schwerin, DE	403	30	1.1	Anestrous	3.10.2000	hCG	3.2	No luteal activity
La Palmyre, F	767	19	1.1	Anestrous	9.1.2001	hCG	CL 3.7	30
La Palmyre, F	767	20	1.1	Anestrous	13.11.2002	hCG	3.8	No luteal activity
La Palmyre, F	767	23	1.1	Anestrous	9.5.2005	hCG	3.5	30
Munster, DE	967	12	1.2	Anestrous	27.1.2002	hCG	3.4	No luteal activity
Whipsnade, UK	1047	14	2.6	Anestrous	12.9.2008	GnRH biorelease	3.4	70
Bewdley, UK	1062	15	1.3	Anestrous	5.11.2007	GnRH biorelease	3.2	70
Cambridge, CA	1193	12	2.3	Anestrous	10.1.2007	GnRH biorelease	n.d.	No luteal activity
Cambridge, CA	1193	12	2.3	Anestrous	10.1.2007	GnRH biorelease	3.4	No luteal activity
Cambridge, CA	1194	12	2.3	Anestrous	18.10.2007	GnRH biorelease	3.2	30
Cambridge, CA	1194	12	2.3	Anestrous	18.10.2007	GnRH biorelease	3.2	No luteal activity
Selwo, ES	1415	9	1.1	Anestrous	30.11.2007	GnRH biorelease	3.3	70
Madrid, ES	1416	10	1.2	Anestrous	28.11.2007	GnRH biorelease	3.4	Pregnant
Cambridge, CA	1193	13	1.3	Anestrous	13.6.2008	GnRH implant	3.7	70
Arnheim, NL	907	21	2.2	Estrous	18.9.2008	GnRH implant	CL 5.5	30
Bratislava, SK	1154	24	1.2	Estrous	14.1.2008	GnRH implant	3.8	70
Bratislava, SK	1154	24	1.2	Estrous	24.9.2008	GnRH implant	3.8	70
Madrid, ES	1416	13	1.2	Anestrous	20.10.2010	GnRH implant	3.5	No luteal activity
Selwo, ES	1415*	11	1.1	Anestrous	19.5.2009	GnRH implant	3.7	Pregnant
Amneville, F	1444	10	1.2	Anestrous	29.6.2010	GnRH implant	n.d.	70
Amneville, F	1445	10	1.2	Anestrous	29.6.2010	GnRH implant	3.4	30
Colchester, UK	1457	10	1.2	Estrous	6.5.2008	GnRH implant	n.r.	30
Colchester, UK	1457	11	1.2	Estrous	7.4.2009	GnRH implant	n.r.	70
Copenhagen, DK	T18	12	2.2	Anestrous	17.10.2008	GnRH implant	n.r.	70
Copenhagen, DK	T18	14	2.2	Estrous	22.9.2010	GnRH implant	3.6	70
Copenhagen, DK	T18	15	1.2	Anestrous	6.5.2011	GnRH implant	3.4	70
Copenhagen, DK	T19	7	2.2	Anestrous	10.8.2010	GnRH implant	3.7	70
Copenhagen, DK	T19	8	1.2	Anestrous	6.5.2011	GnRH implant	3.8	70

c. haem., corpus haemorrhagicum; CMA, chlormadinone acetate; n.d., not detected; n.r., not recorded.
* Died at 30 days post AI with emrbyonic strucure in the uterus.

2.2. Hormone analysis

To evaluate treatment success fecal samples were collected twice per week prior to, throughout and following the estrous induction treatment. All samples were stored at −20°C until analyzed. Fecal 20-oxo-pregnane concentration was analyzed with a group-specific enzyme-immunoassay as described previously [1,9,11]. In two females, blood serum analysis twice per week was performed instead of fecal hormone analysis as part of the routine health check. Blood samples were collected from the leg vein. Plasma progesterone concentrations were determined via EIA using a previously published slightly modified protocol [12,13].

2.3. Statistical analysis

Comparison between treatments was performed with GraphPad InStad (Version 3.00, La Jolla, CA, USA). A contingency table was used to evaluate variances between treatment groups. Comparison between treatment groups was done by χ^2 test with P values <0.05 considered significant. All other values are given as means ± SEM.

3. Results

After ovulation induction an ovarian response, as monitored by ultrasonography, was detected in 93.1%

of the treatments (27/29, Table 1). One day, after ovulation induction a preovulatory sized follicle measuring 3.5 ± 0.05 cm was present in 82.8% of treatments (24/29, Table 1), whereas in 10.3% a fresh corpus luteum of 5.1 ± 0.7 cm had formed (3/29). In 6.9% of treatments no functional structure was detected on the ovary (2/29).

Elevated fecal pregnane or plasma progesterone concentrations 10 days after ovulation induction indicated that chlormadinone combined with hCG or deslorelin, succeeded in inducing ovulation. The overall ovulation rate in all treated females was 77.1% (27/35, Table 1). Of the three different triggers used to induce ovulation, GnRH analogue implants were the most efficient. The ovulation rate of 93.3% using GnRH implants was significantly higher ($P < 0.05$) then ovulation rates achieved by hCG (66.7%) or injectable GnRH analogue (60.5%). HCG was used in one female 6 times to trigger ovulation. Despite sometimes short intervals between hCG applications of 7 or 9 mo this female ovulated 5 out of 6 times after estrous induction.

Ovulation rate in cyclic females treated with GnRH implants was 100% (6/6) compared with 89% (8/9) in anovulatory females. Despite the presence of a preovulatory sized follicle after CMA treatment 33.3%, 37.5% and 6.7% of the females treated with hCG, GnRH or GnRH implants, respectively, failed to ovulate as fecal pregnane concentrations remained at baseline levels (below 100 ng/g feces).

The missing presence of a preovulatory sized follicle or corpus luteum 1 day after ovulation induction in two females was not a clear indicator for the failure of estrous induction. In one female, the increase of fecal pregnane concentration suggested that ultrasound was performed after the ovulatory follicle had ruptured and prior to the formation of a corpus luteum. However, in the other female CMA treatment failed to induce any ovarian response and pregnane concentrations remained at baseline levels. Because of logistical problems, ultrasound was not performed in three of the treated animals (Table 1).

The length of elevated luteal activity when induced with hCG was primarily 4 wk (85.7%, Fig. 1). Luteal activity when induced with GnRH analogue was predominantly 10 wk long (injectable GnRH: 60%; GnRH implants: 71.4%; Fig. 2). The first 3 wk of luteal activity pregnane concentration was similar for both types of luteal activity. In females with a short estrous cycle in response to ovulation induction, pregnane concentration started to cease after 3 wk. In females with a long estrous cycle, pregnane concentration further increased to slowly start ceasing only after 5 wk (Fig. 2). Two anovulatory females, one treated with GnRH implants the other with GnRH injection became pregnant. One of these females carried an embryonic vesicle

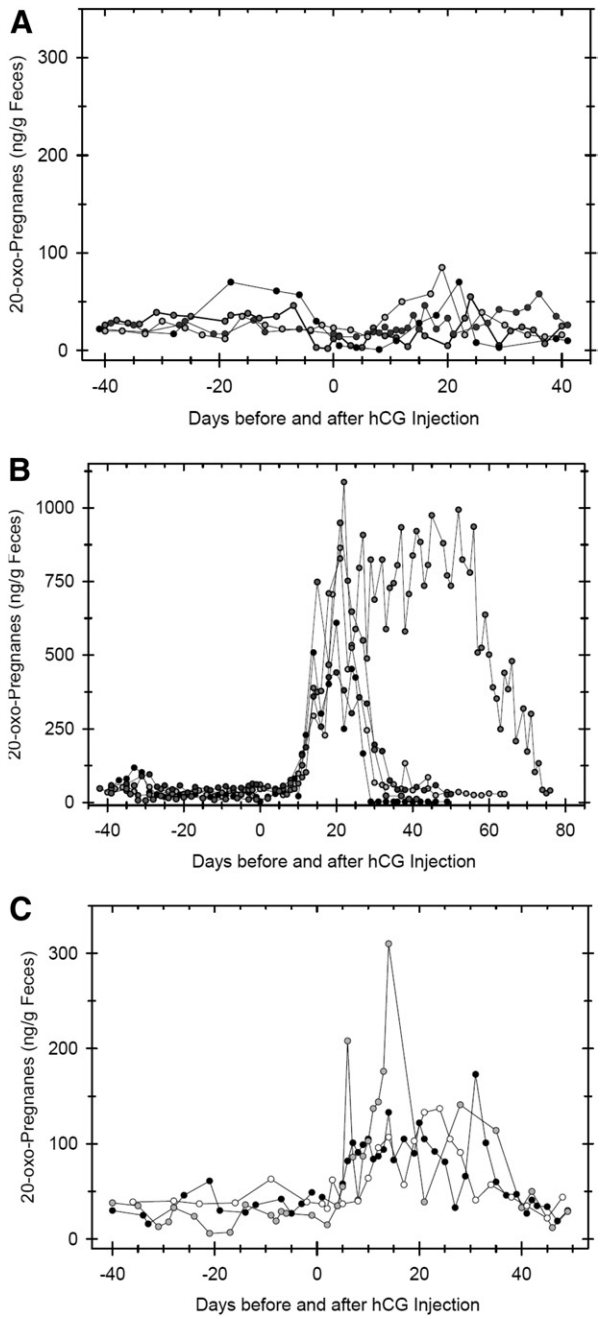

Fig. 1. Fecal pregnane concentration of white rhinoceros from 12 estrous inductions using CMA and hCG. (A) In four treatments luteal activity remained at baseline. (B) In eight treatments luteal activity increased after ovulation induction and ceased after 4 wk except for one. (C) In three females luteal response was less pronounced.

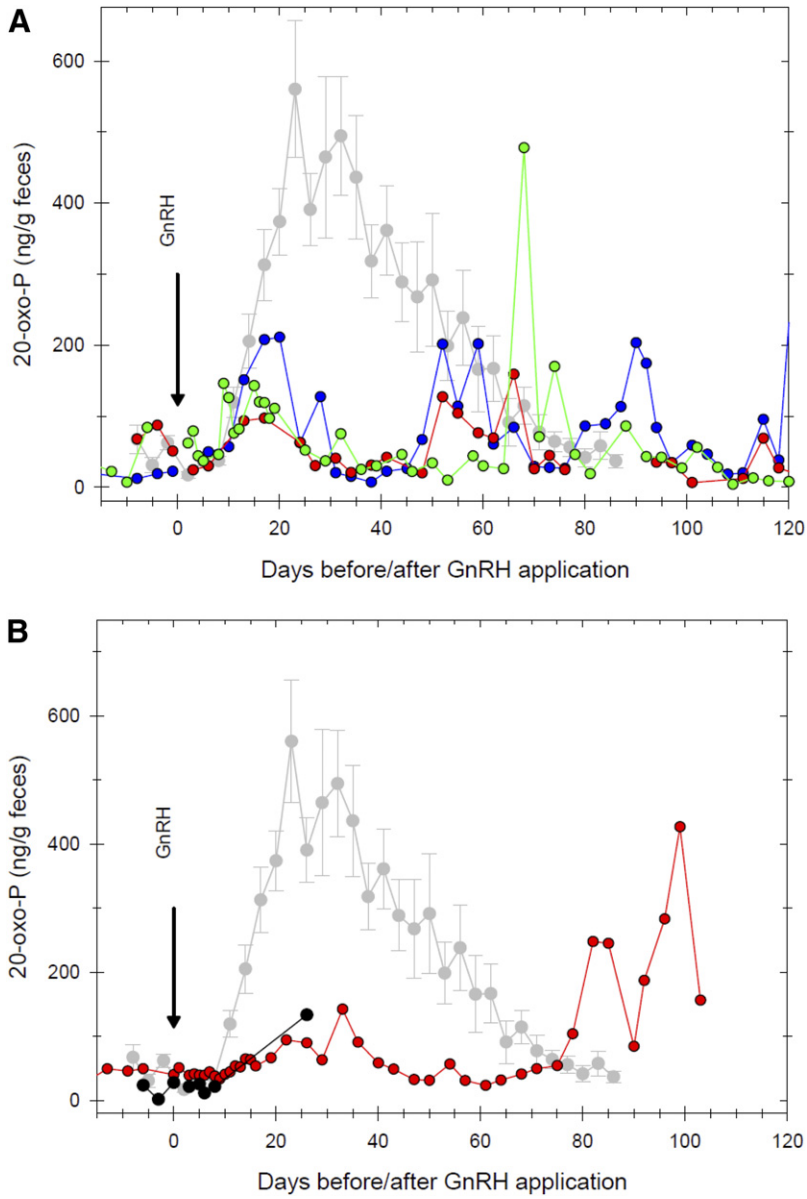

Fig. 2. Fecal pregnane concentration of white rhinoceros from 15 estrous inductions using CMA and GnRH analogue implants: pregnane increases 10 days after ovulation induction. (A) Luteal activity remains elevated for 10 wk (mean ± SEM) or for 4 wk post ovulation induction; in the animals with the 4-wk cycles the induced luteal activity continued. (B) A pronounced increase of pregnane concentration in one pregnant female occurred 60–80 days post ovulation induction; samples from another pregnant female were collection for approximately 4 wk after conception.

when she died 4 wk after ovulation of an unrelated cause. The other carried the pregnancy to term. Interestingly, pregnane concentration in the pregnant female was above baseline but clearly elevated only two mo after estrus induction (Fig. 2). In four of five cycling females regular luteal cycle activity resumed after the CMA treatment. Two of the anovulatory females started displaying spontaneous estrous cycles following estrus induction treatment (Table 1, Fig. 2).

4. Discussion

Mature, healthy, but anovulatory captive white rhinoceroses represent one of the greatest challenges for captive breeding programs in this species [1,2,5]. The principle of estrus synchronization in a rhinoceros was first reported more than 15 yr ago [1,8]. When this initial treatment protocol was repeated in anovulatory rhinoceroses 10 yr later, the success rate for inducing

ovulation was 30% [6]. In this study, the ovulation rate using chlormadinone acetate with hCG or deslorelin for ovulation induction was over 77%, significantly improving the efficiency of estrus induction for this species. Key improvement of estrous induction was the extended period of 9.5 days after the completion of the CMA treatment before ovulation was induced. The use of synthetic GnRH analogue implants replacing hCG and injectable GnRH analogue as ovulation inducer further improved ovulation rate to over 93%. The combination of CMA treatment with deslorelin implants provided a highly efficient and accurate estrous induction protocol for the white rhinoceros. The sustained release of GnRH analogue using implants was more effective compared with the liquid formulation suggesting a better bioavailability of the GnRH in the rhinoceros when slowly released from implants.

Although antibody production against the human Choriogonadotropin has not been tested in this study, multiple use of hCG seemed not to influence the efficacy to induce ovulation in the white rhinoceros. One female ovulated five out of six times when induced with hCG with as short as 7 mo between inductions. Other females did not respond at first but in subsequent inductions. The glycoprotein, hCG, with the human specific β-subunit might rather lack specific receptor binding properties in the rhinoceros, necessary to induce ovulation at a higher rate.

White rhinoceroses are monovulatory. Follicles grow in waves, from which one dominant preovulatory sized follicle develops [3,6,9,11]. In anovulatory females the fate of this preovulatory follicle is atresia or hemorrhage with fecal progesterone metabolites concentrations remaining at baseline [3]. The sustained release of the GnRH analogue deslorelin acetate has previously been shown to induce ovulation in anovulatory and postpartum white rhinoceroses [9,11]. Yet, in these previous studies the development of the dominant follicle to preovulatory size was closely monitored by ultrasound before ovulation was induced. Different from this setting, most anovulatory white rhinoceros are not conditioned for ultrasound monitoring of ovarian events. For the estrus induction protocol described here, folliculogenesis was not closely monitored by ultrasound prior treatment. The synthetic progesterone, CMA, temporarily suppressed the folliculogenesis. The length of synthetic progesterone treatment was calculated to last longer than the physiological life time of a corpus luteum in cycling females. After ceasing the CMA application, folliculogenesis resumed and a preovulatory sized follicle developed within 9.5 days. Ovulation was then triggered by hcG or GnRH inducing luteal activity.

In this study, CMA combined with hCG or GnRH analogue deslorelin reliably produced an ovarian response documented by the presence of the preovulatory sized follicle or corpus luteum one day after ovulation induction, irrespective of the luteal activity at the start of the treatment. The presence and size of a preovulatory sized follicle one day after ovulation induction or 9.5 days after the end of the 45-day chlormadinone treatment was in accordance with previous reports on ovulation induction without a preceding oral synthetic progestin treatment [3,6,9,11]. Between the three triggers used to induce ovulation the slow release GnRH analogue implant achieved a significantly higher ovulation rate and was therefore superior in triggering ovulation compared with hCG and the oily suspension of deslorelin acetate. However, most of the treated females were difficult to approach for a hand injection. In this regard, the remote delivery of the implants in a specific implant-dart [10] was less convenient than the remote delivery of the water soluble hCG or the oily GnRH analogue suspension in a standard blow dart.

Pregnane concentration increased markedly approximately 10 days post ovulation. In animals with 4 wk of luteal activity the pregnane concentration started to regress 3 wk after estrous induction. In animals with 10 wk of luteal activity pregnane concentration started to slowly regress after 5 wk. In pregnant females, a clear increase of pregnane concentration was observed two mo after ovulation. This delay in gestational increase of pregnane concentrations is consistent with previous reports for the white rhinoceroses [2,9,11] and is similar to results found in black [14,15] and greater one-horned rhinoceroses [16]. This suggests that fecal pregnane metabolite excretion during early pregnancy might be different as compared with nonfertile estrous cycles. In this regard, plasma progesterone concentration provides more diagnostic value for pregnancy determination as progesterone concentration after conception rises without delay [9,11].

In the wild, courtship and mating behavior is obvious and well described [17]. In captivity, behavioral estrus in female white rhinoceros is more cryptic and occurs mostly unnoticed by animal managers. An experienced male in the captivity can detect estrous females by showing behavioral interest. However, introduction of territorial males to females without luteal activity can be associated with severe aggression or the death of the female, if the female has ceased her folliculogenesis and does not produce preovulatory sized

follicles due to age or pathologic lesions in the reproductive tract [17]. Most males in captivity however are inexperienced, non-proven breeders that often do not respond to females in (silent) heat or females with regular estrous cycle activity. This greatly hampers breeding management and reproductive success in captivity. Estrus induction following the protocol described in this study will allow for more accurate timing of natural breeding attempts in both, regular cycling and females without luteal activity. Access of the male to the female can be restricted to the receptive period, occurring 9 to 11 days after the completion of the progestin treatment.

Our results show that estrus and ovulation was reliably and timely induced in anovulatory and regular cycling females. Females showing regular estrus prior to the treatment mostly continued with estrous cycle activity after treatment, demonstrating the safety of the protocol. Failure to resume the cycle in one female was possibly influenced by shortening daylight during the winter time. This female had a previous record of cessation of reproductive cyclicity during the winter months. Treatment with CMA and GnRH analogue in two anestrous females stimulated subsequent regular spontaneous estrous cycles overcoming anestrous for the duration of the study.

This is the first report of hormone treatments that initiated regular cyclical reproductive activity in anovulatory white rhinoceros. In the majority of cases, estrus induction failed to induce a lasting effect. Further use and improvements of the estrus induction protocol are warranted in order to initiate regular, spontaneous reproductive activity in captive, anestrous, female white rhinoceros.

Acknowledgments

The authors are grateful to all helpful staff, as well as for all logistical and financial support provided by the participating European and Canadian Zoos contributing to this study. In addition, the authors are thankful for technical support by Nga Nguyen, Andrea Krause, Imke Lueders, and Jette Dierich, as well as to Joseph Saragusty for editing the manuscript.

References

[1] 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 rhinoceroses (Ceratotherium simum). Anim Reprod Sci 1998;53:173–90.

[2] Patton ML, Swaisgood RR, Czekala NM, White AM, Fetter GA, Montagne JP, et al. Reproductive cycle length and pregnancy in the southern white rhinoceroses (Ceratotherium simum simum) as determined by faecal pregnane analysis and observations of mating behavior. Zoo Biol 1999;18:111–27.

[3] 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.

[4] 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.

[5] Versteege L. White Rhinoceros European Endangered Species Program Annual Report 2006. Safaripark Beekse Bergen, Hilvarenbeek, Netherlands, 2007.

[6] 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.

[7] Godfrey RW, Pope CE, Dresser BL, Bavister BD, Andrews JC, Olsen JH. An attempt to superovulate a southern white rhinoceros (Ceratotherium simum simum). Theriogenology 1990;33:231.

[8] Walzer C, Schwarzenberger F. Estrous induction in a white rhinoceroes (Ceratotherium simum simum) and concomitant EIA fecal progestagen analysis. Proceedings of the American Association of Zoo Veterinarians, East Lansing, MI, USA. 1995;322–4.

[9] 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;67:1473–84.

[10] Walzer C, Silinski S, Hermes R, Göritz F, Hildebrandt T, Schwarzenberger F. Remote delivery dart for hormonal implants in rhinoceros. Proceedings of the American Association of Zoo Veterinarians, San Diego, CA, USA. 200;642–3.

[11] Hermes R, Göritz F, Saragusty J, Sós E, Molnar V, Reid CE, et al. First successful artificial insemination with frozen-thawed semen in rhinoceros. Theriogenology 2009;71:393–9.

[12] Graham L, Schwarzenberger F, Moestl E, Galama W, Savage A. A versatile enzyme immunoassay for the determination of pregestogens in feces and serum. Zoo Biol 2001;20:227–36.

[13] Lueders I, Niemuller C, Gray C, Rich P, Hildebrandt TB. Luteogenesis during the estrous cycle in Asian elephants (Elephas maximus). Reproduction 2010;140:777–86.

[14] Schwarzenberger F, Francke R, Göltenboth R. Concentrations of fecal immunoreactive progestagen metabolites during the estrous cycle and pregnancy in the black rhinoceros (Diceros bicornis michaeli). J Reprod Fertil 1993;98:285–91.

[15] Berkeley EV, Kirkpatrick JF, Schaffer NE, Bryant WM, Threlfall WR. Serum and fecal steroid analysis of ovulation, pregnancy and parturition in the black rhinoceros (Diceros bicornis). Zoo Biol 1997;16:121–32.

[16] Kasman LH, Ramsay EC, Lasley BL. Urinary steroid evaluations to monitor ovarian function in exotic ungulates: III. Estrone sulfate and pregnanediol-3-glucuronide excretion in the Indian rhinoceros (Rhinoceros unicornis). Zoo Biol 1986; 5:355–61.

[17] Hutchins M, Kreger MD. Rhinoceros behaviour: implications for captive management and conservation. Int Zoo Yearbook 2006;40:150–73.