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RESEARCH ARTICLE

Use of Urinary Biomarkers of Ovarian Function and Altrenogest Supplementation to Enhance Captive Breeding Success in the Indian Rhinoceros (*Rhinoceros unicornis*)

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Urinary hormone analysis was conducted on two adult female Indian rhinoceroses (*Rhinoceros unicornis*) that exhibited minimal or no estrual behaviors traditionally used to time breeding. Urine was collected throughout two consecutive estrous cycles to establish preliminary data on each individual's pattern and concentration of estrogen conjugates (EC) and progesterone metabolites (PdG) during follicular and luteal phases. Following preliminary endocrine analysis, urine samples were shipped on a frequent basis to verify when each female was off baseline in EC. Estrus and breeding dates were then predicted. Females were introduced to fresh male rhinoceros fecal samples daily throughout the follicular phase to potentially stimulate estrous behaviors. Despite successful assessment of follicular phase dynamics, females sometimes failed to exhibit estrus. Both females conceived following mating introductions that were timed using hormone analysis. Pregnancy was diagnosed either by endocrine analysis or rectal ultrasonography. Progestational support (altrenogest) occurred after pregnancy confirmation and varied for each female (21 and 66 days post-breeding). One female experienced early pregnancy loss and the other successfully completed a term pregnancy. These results demonstrate that a science based management strategy that relies on urinary biomarkers of ovarian function can facilitate naturally breeding captive Indian rhinoceroses. Zoo Biol. 33:83–88, 2014. © 2013 Wiley Periodicals, Inc.

Keywords: pregnancy; estrous cycle; estrogen conjugates; progesterone metabolites; silent estrus; progesterone supplementation

INTRODUCTION

The female Indian rhinoceros (*Rhinoceros unicornis*) typically exhibits overt signs of estrous behaviors that have been traditionally used to time introductions for mating [Lang, 1972; Lang, 1975; Tong, 1961]. Even so, some male Indian rhinoceroses have failed to sire offspring because they expressed significant aggression when introduced to a behaviorally estrual female [Gomez et al., 2004]. These aggressive interactions have limited the number of breeding pairs, resulting in a captive population over-represented by a few highly prolific founders [Zschokke et al., 1998; Foose and Wiese, 2006].

The captive Indian rhinoceros population consists of 189 animals distributed across 70 different zoological institutions throughout North America, Europe, Asia and Australia [von Houwald, 2012]. Wild population estimates for the species currently range from 2,800 to 2,850 individuals [http://www.rhinos.org/rhinos/greater-one-horned-rhino]. Regular breeding between a rhinoceros pair is not only important

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to the genetic health of the population, but also to the long-term reproductive health of the female. Many reproductive problems in captive female rhinoceroses are directly attributed to prolonged periods of estrous cyclicity without breeding and/or pregnancy [Jones, 1979; Hermes et al., 2004, 2006]. Therefore, missed breeding opportunities can negatively impact the future fertility of the female rhinoceros.

Longitudinal ultrasound, behavior and endocrine evaluations have been conducted over extended periods of time in the female Indian rhinoceros to characterize the estrous cycle and pregnancy [Kassam and Lasley, 1981; Kasman et al., 1986; Schwarzenberger et al., 2000; Gomez et al., 2004; Stoops et al., 2004]. Results from these studies helped elucidate some of this species' reproductive characteristics largely due to the integration of ultrasonography [Stoops et al., 2004] with more traditional methods used to study reproductive function. As a result, several biomarkers of ovarian function have been determined for the Indian rhinoceros and include (1) a 14-day rise in estrogen conjugate concentrations (EC) during the follicular phase, (2) a transient spike in progesterone metabolite (PdG) concentrations prior to ovulation, and (3) a 17–21 day elevation in PdG concentrations throughout the luteal phase.

The aim of this study was to use urinary EC and PdG analysis to predict estrus and time mating introductions for Indian rhinoceroses at remote facilities to enhance breeding success. Females were exposed to fresh male rhino fecal samples during the follicular phase to potentially stimulate estrous behaviors in females with a history of silent estrus. Hormone analysis and ultrasonography were employed to diagnose and monitor resulting pregnancies. Lastly, we examined progestational support of gestation by intervention with altrenogest.

MATERIAL AND METHODS

Animals and Sample Collection

This research was conducted on two female Indian rhinoceroses maintained at the Oklahoma City Zoo, Oklahoma City, OK, USA and Fort Worth Zoo, Fort Worth, TX, USA. Female SB161 was captive born at the Bronx Zoo on September 5, 1989 and transferred to the Oklahoma City Zoo November 8, 1993. Female SB161 gave birth to two calves (1996 & 2001) after gestation lengths of 473 and 484 days before experiencing a spontaneous abortion during her third pregnancy (2003) at 211 days of gestation. The same male (SB125) sired all calves and pathology results on the aborted fetus/placenta were non-specific (G. West, personal communication). While SB161 had historically shown overt signs of estrus, she failed to consistently demonstrate these behaviors following pregnancy loss. Female SB161 was 14–16 years of age during the study. Urine samples were collected from SB161 over 546 days from July 28, 2004 to January 25, 2006. The second female, SB191, was wild caught in 1989 and transferred to the Fort Worth Zoo 10 May 1990. The female had been unsuccessfully paired with a male prior to the 2001 transfer of a proven male (SB106). As SB191 exhibited minimal to no estrual behaviors, timing breeding introductions proved difficult. Female SB191 was 14–15 years of age during the study. Urine samples were collected from SB191 over 429 days from June 9, 2004 to September 1, 2005.

Fresh urine samples were aspirated off the stall floor or collected directly into a storage vial. Samples were stored frozen (−20°C) following collection and shipped on ice packs to the Center for Conservation and Research of Endangered Wildlife (CREW). Urine was collected throughout two consecutive estrous cycles to establish preliminary data on each individual's pattern and concentration of EC and PdG during follicular and luteal phases. The number of days urinary EC remained elevated above baseline was determined and the pattern of PdG excretion during the late follicular phase was examined for any transient rise indicative of ovulation [Stoops et al., 2004]. Finally, the concentration and excretion pattern of PdG and EC during the follicular to luteal transition was determined. Data from the two cycles were used to calculate an interovulatory interval. Following initial analysis, urine samples were shipped on a frequent basis to verify when each female was off baseline in EC. Estrus and breeding dates were then predicted. Females were introduced to fresh male rhino feces daily during the follicular phase to potentially stimulate estrous behaviors [Stoops et al., 2004]. Behavioral signs of estrus were reported by keeper staff of the respective zoos', and this assessment was based on the keepers knowledge of the individual animals. These behaviors included a decrease in appetite, increased activity pattern, whistle vocalization, difficulty in shifting, increased urination frequency and urine squirting. Pairs were introduced for breeding at the discretion of keeper, curatorial and veterinary staff of each zoo. If the male and female did not breed, urine was analyzed to characterize the estrous cycle in an attempt to explain mating failure. If breeding did occur, urine was analyzed at 30 days post-breeding to determine possible pregnancy status. If urinary PdG concentrations indicated an extended luteal phase, urine was shipped again at day 50 to verify a pregnancy diagnosis.

Ultrasonography

During the course of the study, female SB191 was conditioned to allow rectal ultrasound examinations [Stoops et al., 2004]. The ultrasound machine was an Aloka 500 V (Aloka, Wallingford, CT) with a 3.5 mHz convex or 5 mHz liner probe. Rectal ultrasound examinations were performed Days 17–21 post-breeding to detect pregnancy in 2005.

Enzyme Immunoassays

Concentrations of urinary EC and PdG were measured using EIA techniques described by Munro et al. [1991]. Polyclonal antibodies R5222 and R13904 (supplied by C. Munro, University of California, Davis, CA) were utilized in

the EC and PdG EIA's, respectively. Standard concentrations for the EC assay ranged from 3.9 to 250 pg/well, whereas the PdG top standard extended to 500 pg/well. Creatinine concentrations were measured using a commercially available kit (no. 558-A; Sigma Diagnostics, St. Louis, MO). Urine samples were diluted (1:20) in ddH_20 for the creatinine assay. Urinary concentrations of steroids were indexed by creatinine concentration and expressed as ng/mg creatinine (Cr). Inter and intra assays of CV were $<15\%$ and $<10\%$, respectively for the two assays.

Statistical Analysis

The Sigma Plot Version 10/Sigma Stat Version 3.5 software program (SPSS, Inc., Chicago, IL) was used for statistical analysis and $P < 0.05$ was considered significant. Standard descriptive statistics were used to summarize results and statistical significance was tested using a Student's *t*-test. All data are presented as mean $\pm$ SD. Baseline and elevated concentrations of EC and PdG were calculated for each female using an iterative process in which values that exceeded 2 SD above the mean were excluded. The average of the remaining values was considered baseline and values greater than twice the SD were considered elevated.

Progesterone Supplementation

The synthetic progesterone altrenogest (Regu-Mate Intervet, Inc., Millsboro, DE) was administered daily following ultrasonographic confirmation of pregnancy in SB191 until day 112 post-breeding when pregnancy loss was confirmed by urinary hormones and ultrasonography. Because female SB161 was not conditioned for rectal ultrasonography, altrenogest was administered only following urinary hormone confirmation of pregnancy (Day 66 post-breeding). A standard equine dosage was prescribed, with body weight estimated at 2,000 kg for both females (40 mL v/v). Only female SB161 completed a term pregnancy. Beginning on day 445 of gestation, altrenogest dosage was decreased by 2 mL/day. The female was no longer receiving the supplement by day 465 of gestation. It was discovered that for 2 days/week from Day 66–270 of gestation, female SB161 received a higher dose (60 mL vs. 40 mL) of altrenogest. Upon discovery of the discrepancy, SB161 was weaned down to a 40 mL dosage for those 2 days of the week (5 mL/day over 2 weeks). To ensure the entire dosage was administered to both females, altrenogest was mixed in peanut butter or banana paste before being spread onto slices of bread and fed to the female.

RESULTS

Preliminary Estrous Cycle Analysis

Concentrations of urinary EC excreted during the follicular phases of SB161 and SB191 did not differ ($P = 0.93$). However, luteal phase PdG concentrations in female SB191 were ten-fold lower ($P = 0.008$) than those excreted by SB161. Both females exhibited estrous cycles at regular intervals (SB161, 43 days; SB191, 46–53 days).

Female SB161

Mean urinary EC during the follicular phase ($n = 3$ cycles) was 2539.87 ± 2333.92 ng/mg Cr. Urinary PdG concentrations averaged 704.30 ± 829.79 ng/mg Cr during the luteal phase ($n = 2$ cycles). Female SB161 exhibited strong estrual behavior within 3 days of predicted estrus and conceived on the first cycle in which urinary hormone analysis was used to time breeding. She gave birth to a healthy live female calf (SB369) after 491 days of gestation, 26 days after her last dose of exogenous progesterone supplementation. PdG concentrations returned to baseline shortly following birth (Fig. 1). Urine samples collected on Days 2 and 8 post-partum had PdG concentrations measuring 228.73 and 70.45 ng/mg Cr, respectively. Between Days 445–465 (interval during which altrenogest dosage was decreased by 2 mL/day) urinary PdG concentrations averaged 4155.0 ± 1471.09 ng/mg Cr, and did not differ ($P = 0.205$) from concentrations excreted 20 days earlier (Days 424–444, 5458.8 ± 1748.84 ng/mg Cr) (Fig. 1). Similar results were observed in regards to urinary EC concentrations ($P = 0.644$; Days 424–444, 73.1 ± 32.41 ng/mg Cr; Days 445–465, 84.9 ± 48.65 ng/mg Cr). Concentrations of PdG during the first 6 months of gestation were comparable to those excreted during the luteal phase (Fig. 1). Thereafter, PdG concentrations steadily increased (Fig. 1). Urinary EC concentrations remained low throughout pregnancy (Fig. 1).

Female SB191

A total of nine estrous cycles were monitored during the study to time breeding introductions. Mean EC during the follicular phase ($n = 9$ cycles) was 2611.158 ± 1724.99 ng/mg Crt. Concentrations of PdG averaged 77.363 ± 729.05 ng/mg Crt during the luteal phase ($n = 7$ cycles). In five of nine estrous cycles, SB191 exhibited estrous behaviors significant enough to warrant introduction to the male. During four of these cycles (cycle #1, 2, 4, 9), successful mating occurred. A transient rise in PdG (73.76 ± 2.12 ng/mg Cr; $n = 4$ cycles) was detected 1 day post-breeding. During the one cycle (#6) in which mating did not occur even though estrus was exhibited, inclement weather and a disinterested male inhibited efforts to successfully introduce the pair. Actual estrus occurred 1–6 days later (cycle #1, 2, 4, 6) and 3 days earlier (cycle #9) than predicted estrus. Of the four remaining estrous cycles monitored during the study, SB191 exhibited minimal ($n = 1$, cycle #8) to no ($n = 3$, cycle #3, 5, 7) estrous behaviors regardless of having olfactory exposure to the male. Urinary hormone analysis suggested a possible pregnancy following cycle #2 of the study (Fig. 2A), as PdG remained elevated beyond a normal luteal phase. At the time, SB191 was not conditioned for rectal ultrasonography

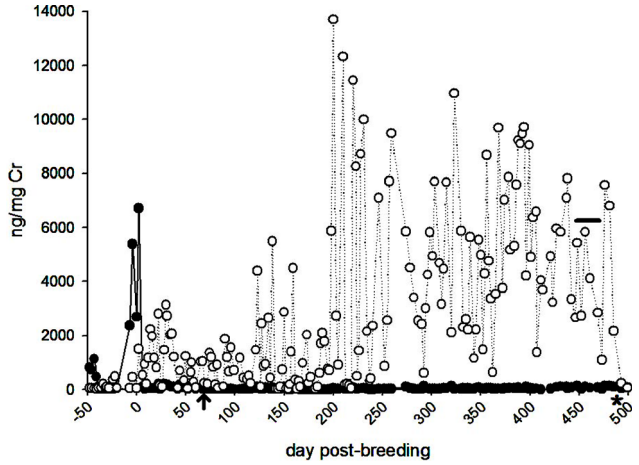

Fig. 1. Urinary estrogen conjugate (EC, ■) and progesterone metabolite (PdG, ◯) concentrations throughout gestation in female Indian rhinoceros SB161 aligned from day of estrus and breeding. Arrow denotes start of altrenogest treatment. The black horizontal bar indicates the 20 days that altrenogest was decreased by 2 ml/day and the star indicates date of parturition.

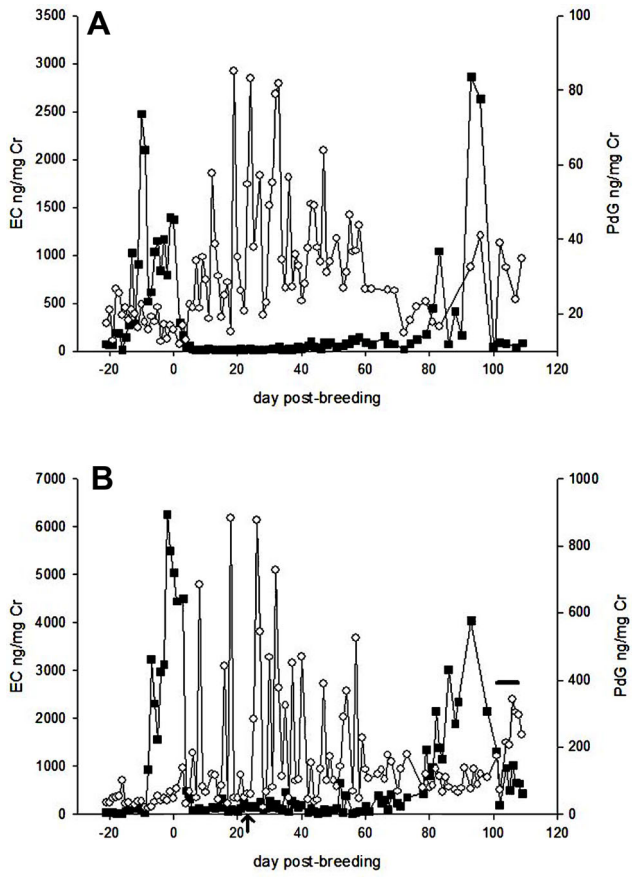

Fig. 2. Urinary estrogen conjugate (EC, ■) and progesterone metabolite (PdG, ◯) excretion profile in SB191 during a (**A**) suspect and (**B**) confirmed pregnancy. The arrow in (B) denotes start of altrenogest treatment, and the black horizontal bar at Day 112 post-breeding indicates end of altrenogest treatment.

and pregnancy could not be confirmed. Therefore, she was not administered supplemental progesterone. However, the female was eventually conditioned for ultrasound, and pregnancy was confirmed 21 days after breeding (cycle #9) through measurement of a 2.5 cm embryonic vesicle in the uterus (Fig. 3). Urinary PdG concentrations throughout the first 20 days after breeding in the suspect and confirmed pregnancies were 29.45 ± 17.37 and 177.39 ± 242.39 ng/mg Cr, respectively. In both cases, abortion appeared to occur before Day 80 of gestation (Fig. 2A,B). The female was still receiving a full altrenogest dosage throughout the first estrous cycle following pregnancy loss (Fig. 2B). The follicular phase that took place during altrenogest treatment was longer in length (>20 days) than previous estrous cycles, and the female failed to exhibit estrus (Fig. 2B).

DISCUSSION

Urinary EC and PdG analyses were effective in predicting estrus and breeding in female Indian rhinoceroses that had been difficult to manage for mating introductions using traditional means. While fecal, urine and salivary hormone evaluations have been conducted on Indian rhinoceroses to characterize the estrous cycle and pregnancy [Kassam and Lasley, 1981; Kasman et al., 1986; Schwarzenberger et al., 2000; Gomez et al., 2004; Stoops et al., 2004], this is the first study to employ a prospective approach to endocrine analysis in order to promote successful breeding. We collected urine, as opposed to feces, in order to more accurately match real time systemic hormone events.

The female Indian rhinoceros typically exhibits overt estrous behaviors compared to other rhinoceros species [Tong, 1961; Lang, 1972; Lang, 1975; Stoops et al., 2004]. These behaviors are used alongside knowledge of last estrus to time introductions for mating. Estrous cycle length is quite

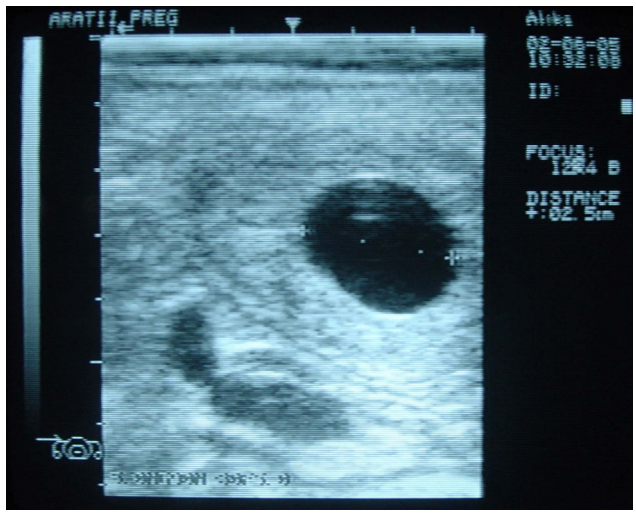

Fig. 3. Ultrasound image of an embryonic vesicle in Indian rhinoceros SB191 at 21 days post-breeding.

variable in this species [Kassam and Lasley, 1981; Kasman et al., 1986; Schwarzenberger et al., 2000; Gomez et al., 2004; Stoops et al., 2004] and females can display single and split estrus behavior [Stoops et al., 2004]. The two female Indian rhinoceroses in our study either failed to exhibit or showed minimal estrual behaviors. To overcome this challenge, we used intensive endocrine analysis to predict estrus and time breeding introductions. Females were exposed to fresh male feces during the follicular phase in the hopes it would aid in exhibition of estrus. Even though follicular phase dynamics were accurately predicted and olfactory cues received, one female failed to exhibit estrus in some of the cycles monitored. Lack of estrous behavior in the female African white rhinoceros is often indicative of an anoestrous state [Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001], but did not appear the case for Indian rhinoceroses in our study. Silent estrus occurs in many domestic [Cummings, 1942] and other exotic species [Lindburg and Fitch-Snyder, 1994], but this is the first report in an Indian rhinoceros. Mate preference is implicated in behavioral manifestation of estrus in the white rhinoceros [Patton et al., 1999]. However, it does not appear to be a primary factor for the lack of behavior we observed, as estrus and breeding took place prior to and subsequent of silent estrus cycles. In mares, silent estrus appears to be related to changes in estrogen production and pattern of secretion as related to regression of the corpus luteum [Nelson et al., 1985]. Similar hormone dynamics may be responsible for this phenomenon in the Indian rhinoceros.

Luteal insufficiency was suspected in both Indian rhinoceroses in our study. However, it remains unknown if it was the reason for abortion at 6 months of gestation in the preceding pregnancy of the multiparous female, or whether it was a factor during the term pregnancy we monitored. Urinary concentrations of PdG excreted by the nulliparous female were markedly lower than normative values established in this species [Stoops et al., 2004] and provide a stronger case for luteal insufficiency. Despite the low concentrations, this female appeared to ovulate during cycles in which estrus was exhibited, as transient spikes in PdG were measured one day post-breeding. However, diagnosing pregnancy based solely on urinary PdG concentrations proved difficult and ultrasound became critical to a definitive diagnosis. Ultrasonographic detection of early pregnancy in the Indian rhinoceros was similar to the African white [Radcliffe et al., 1997], African black [Berkeley et al., 1997; Radcliffe et al., 2001] and Sumatran [Roth et al., 2001] rhinoceroses, and confirms previous findings for this species [Stoops et al., 2007].

Supplemental progesterone therapy has been used throughout gestation in the African black [Berkeley et al., 1997] and Sumatran [Roth et al., 2004] rhinoceros, resulting in the birth of live offspring. This is the first report of altrenogest supplementation during pregnancy in the Indian rhinoceros. Hormone dynamics throughout gestation were comparable to those observed previously in this species [Kasman et al., 1986]. Similarly, serum progesterone concentrations during a term pregnancy in a Sumatran rhinoceros supplemented with altrenogest did not differ from those during a subsequent pregnancy in the same female, for which no progesterone supplementation was used, and a qualitative rather than quantitative benefit from altrenogest was suggested [Roth, 2006]. The female Indian rhinoceros took longer (26 days) to give birth after final altrenogest dosage compared to the Sumatran [10 days; Roth et al., 2004] and African black [14 days; Berkeley et al., 1997] rhinoceros, and may be due to the longer average gestation in this species [von Houwald, 2012], since dependency on altrenogest this late in gestation is unlikely.

We found no negative effect from administering altrenogest throughout pregnancy in the Indian rhinoceros, but discovered it did not prevent early pregnancy loss in a female that had been excreting low luteal concentrations of PdG. Early pregnancy loss has now been reported now in all captive rhinoceros species [Berkeley et al., 1997; Radcliffe et al., 1997; Patton et al., 1999; Roth et al., 2001]. In mares, embryonic loss between Days 20 and 40 results in maintenance of the CL for approximately 60 days [Ginther, 1995]. Similar findings were reported in the African white rhinoceros [Radcliffe et al., 1997], and our results indicate the same may be true for the Indian rhinoceros. Pyometria and endometritis can contribute to early death of horse embryos [Ginther, 1995], and similar losses have been documented in African white rhinoceroses [Radcliffe et al., 1997; Patton et al., 1999]. One female Indian rhinoceros in our study was administered altrenogest upon confirmation of an embryonic vesicle at Day 21 post-breeding, similar to the timeframe followed for a Sumatran rhinoceros [Day 17, Roth et al., 2004]. Despite initiating progesterone supplementation early in gestation, the female Indian rhinoceros still experienced pregnancy loss. The exact day of embryonic loss remains unknown because the female became uncooperative with ultrasound after initial diagnosis. As a result, she remained on supplemental progesterone and we documented the initiation and completion of an estrous cycle while on altrenogest. The other female Indian rhinoceros in the study successfully completed a term pregnancy on supplemental progesterone. Although she received the initial dose at a later date (66 post-breeding), it was still earlier than when first administered to an African black rhinoceros [Day 144, Berkeley et al., 1997].

Our results demonstrate that a science based management strategy that relies on urinary biomarkers of ovarian function can facilitate natural breeding efforts for captive Indian rhinoceros. The goal of this study was to overcome the problem of timing breeding introductions in Indian rhinoceroses failing to show good behavioral signs of estrus, ultimately to produce pregnancies and live offspring. By integrating reproductive science based on what has been learned to date into the management of captive Indian rhinoceros, breeding success can be enhanced while additional knowledge is gained about the species' reproductive physiology.

CONCLUSIONS

1. Urinary EC and PdG concentrations and pattern of excretion can facilitate breeding introductions in the Indian rhinoceros.
2. Female Indian rhinoceroses can sometimes fail to exhibit behavioral signs of estrus during an estrous cycle.
3. Progestational support (altrenogest) throughout gestation in the Indian rhinoceros does not impact the successful birth of a live calf.
4. Administration of altrenogest starting at Day 21-post breeding in a female Indian rhinoceros with a history of low PdG concentrations did not prevent early pregnancy loss.
5. The female Indian rhinoceros is capable of exhibiting an estrous cycle while receiving a standard equine dosage of altrenogest.

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