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Urinary Steroid Evaluations to Monitor Ovarian Function in Exotic Ungulates: III. Estrone Sulfate and Pregnanediol-3-Glucuronide Excretion in the Indian Rhinoceros *(Rhinoceros unicornis)*

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A study was undertaken to identify urinary estrogen and progesterone metabolites in the female Indian rhinoceros *(Rhinoceros unicornis)*. Measurements of these metabolites were then used to monitor ovarian function and establish normal levels and patterns of steroid excretion during the estrous cycle and pregnancy. Urine samples were analyzed for estrone sulfate and pregnanediol-3-glucuronide (PDG) by direct radioimmunoassays. Both hormones produced discrete profiles reflecting ovarian activity in nonconceptive cycles. The estrous cycle was observed to be 48 days (range 39–64) with a mean follicular phase of 14.8 days (range 13–19), followed by a mean luteal phase of 19 days (range 17–21). Of the single gestation monitored, PDG levels rose above luteal phase levels by the third month after breeding and remained elevated throughout gestation. The combined estrogen and progesterone metabolite profiles present a complete evaluation of ovarian steriod production in the mature female Indian rhinoceros.

Key words: Indian rhinoceros, estrous cycle, pregnancy, urinary hormones

INTRODUCTION

The Indian rhinoceros (*Rhinoceros unicornis*) is an endangered species that has had poor reproductive success in captivity [Ruedi, 1983]. The species' intractability, small captive population size, and infrequent captive breeding have limited our knowledge of its reproductive biology. Additionally, the species' large size and economic value have led to reluctance by owners to transport animals for breeding purposes. To improve captive propagation of the Indian rhinoceros and assure its

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long-term survival, a better understanding of this species' reproductive physiology is essential.

Analysis of urinary steriod metabolites has been shown to be a practical and accurate method for monitoring reproductive function in a variety of mammalian species [Lasley, 1985]. Kassam and Lasley [1981] reported the urinary estrogen excretory profile, correlated to observed estrous behavior, in the Indian rhinoceros. Subsequently, urinary estrone sulfate (ES) analysis has been demonstrated to be a simpler technique for monitoring ovarian follicular activity in primates [Shideler et al, 1983] and perissodactyls [Evans et al, 1984; Kasman et al, 1985]. Loskutoff et al [1983] presented preliminary data indicating pregnanediol-3-glucuronide (PDG) to be a major urinary metabolite of progesterone in a number of species and useful for monitoring corpus luteum function in the Indian rhinoceros.

In the present report, levels of urinary ES and PDG during the estrous cycle and pregnancy in the Indian rhinoceros are described, following their measurement using simplified assay methods. This represents the first report of the concomitant measurement of ovarian estrogen and progesterone metabolite excretion in an endangered ungulate and provides a basis for the practical evaluation of reproductive status of the female Indian rhinoceros.

MATERIALS AND METHODS

Sample Collection

Random urine samples were obtained from six mature female Indian rhinoceros held in four North American zoos. Samples were collected at least three times per week either by midstream catch or by aspiration from the ground and kept frozen until analyzed. Behavioral estrous signs (whistling, increased urination), when observed, were noted by keepers.

Sample Analysis

All urine samples were initially analyzed for creatinine concentration (CR) by the method of Taussky [1954]. Hormone values are presented as mass per milligram CR to adjust for variation in urinary water content.

Urinary ES levels were determined by a direct radioimmunoassay (RIA) as described by Shideler et al [1983]. The antiserum used in this study (LK-2-10) was produced in a rabbit against estrone-3-glucuronide BSA. The antigen was prepared by the method of Conrow and Bernstein [1971], utilizing the immunization protocol of Vaitukaitus et al [1971].

Cross-reactivities of the antiserum to related estrogens on a mass basis using tritiated estrone sulfate (6,7-^{3}H-estrone sulfate, 7,000 cpm, sp. act. 55 Ci/mM, New England Nuclear, Boston) as the standard were estrone (130%), estradiol 17β (10%), estrone glucuronide (110%), estradiol-3-glucuronide (<0.001%). Dilution of the antisera was adjusted to allow 100 μl to bind 40% of the ES tracer (7,000 cpm) in the absence of competing nontritiated ES.

Unprocessed urine samples were serially diluted (1:10 to 1:10,000) with deionized water, and 0.05 ml of the diluted samples was assayed. The interassay coefficient of variation for all ES assays in this study was 7.5% at 29–31% binding (n = 27). The functional sensitivity of the assay based on the lowest dilution was 7.8 ng/ml of urine or 39.0 pg/assay tube.

The PDG RIA was performed by the method of Loskutoff et al [1982] with commercially prepared antibody and tracer (Courtauld Institute of Biochemistry, London). Unprepared urine samples were serially diluted (1:10 to 1:1,000 with deionized water), and 0.01 or 0.05 ml was assayed.

The coefficients of variation for all PDG assays in this study were 13.2% and 15.1% at 73–75% and 29–31% binding, respectively (n = 22). The functional sensitivity of the PDG assay was 7.8 ng/ml urine or 39.0 pg/assay tube.

Assay Validation

Validation of each steroid conjugate was achieved by dose-response analysis and high-performance liquid chromatography (HPLC). Urine samples representing different reproductive states (before and after observed estrus; and early, mid, and late pregnancy) were serially diluted with deionized water and assayed concomitant to serially diluted pure ES or PDG standards in respective assays. Parallel responses of all samples to both hormones were found, indicating their quantifiable presence in Indian rhinoceros urine.

HPLC was performed on representative samples as described by Shideler et al [1983] to identify the possible immunoreactive substances for each of the two assays. Aliquots of the five urine samples described above were combined with tritiated ES and estrone glucuronide or pregnanediol-3-glucuronide and 20 alpha hydroxyprogesterone (2,000–5,000 cpm, each). These markers were selected as being potential urinary metabolites present in Indian rhinoceros urine, and their elution patterns were compared to immunoreactive elution peaks in each sample evaluated. Chromatography of estrone and progesterone metabolites was performed separately on matched urine samples. Aliquots of HPLC eluates were taken for direct counting of tracers, and separate aliquots were assayed for respective immunoreactivity.

All samples revealed a single immunoreactive peak that cochromatographed with the ES tracer. This peak accounted for 95–99% of the total estrone conjugate immunoreactivity measured in matched samples assayed without prior chromatography. Similarly, all samples revealed a single peak consistent with the PDG tracer. This peak accounted for 96–99% of the total immunoreactivity measured in matched samples assayed without prior chromatography. Recovery of tracers following chromatography was 86. 7 $\pm$ 3.33% (n = 10).

RESULTS

Ten estrous periods were monitored—two successive estruses in five individuals. The mean ES concentrations in random urine samples (indexed by creatinine (CR)) around the time of ovulation, are illustrated in Figure 1. The ES profile is aligned at the day of the precipitous decline of ES (D0) which presumably correlates with the collapse of the dominant follicle at the time of ovulation. Baseline ES levels from 25 through 18 days prior to D0 range from 0.02 to 2.0 μg/mg CR ($\bar{X}$ = 0.8 $\pm$ 0.3 μg/ng CR). These levels are followed by an increase of 4.32 $\pm$ 2.2 μg/mg CR/day, 17 through 5 days prior to D0. ES levels plateau at 47.3 $\pm$ 9.05 μg/mg CR from 4 through 1 day prior to D0 (range 3.5–164.0 μg/mg CR), then decline back to respective baseline levels from 1 through 7 days post-D0 at a mean rate of 2.3 $\pm$ 1.7 μgmg CR/day. In six complete follicular episodes monitored, ES levels were greater than respective baseline values for 14.8 days (range 13–19). The mean estrous cycle,

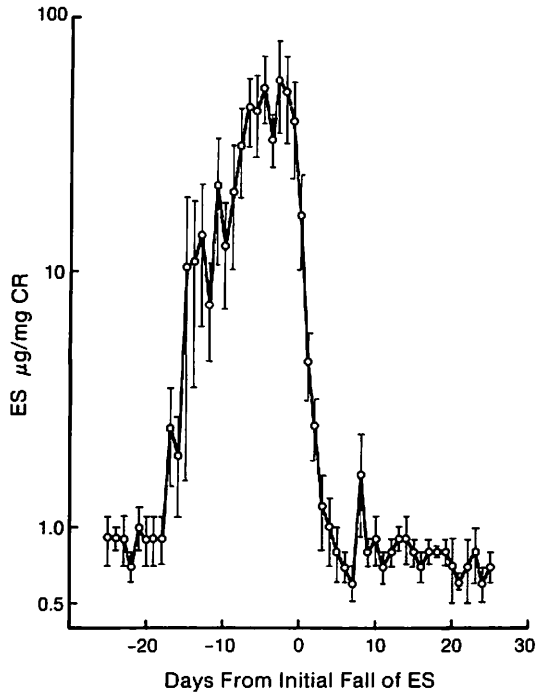

Fig. 1. Immunoreactive urinary estrone sulfate (ES) levels ($\bar{X} \pm$ SEM; n = 10) during the Indian rhinoceros estrous cycle. All values are aligned on the precipitous fall of ES and are indexed by creatinine (CR).

as defined by the inter-D0 interval, was 48 days (range 39–64).

The profile of mean PDG levels in the samples described above is presented in Figure 2. Baseline PDG levels between 23 and 6 days prior to D0 range from 14.3 to 34.4 ng/mg CR ($\bar{X}$ = 20.4 ± 1.3 ng/mg CR). PDG levels begin to rise above this baseline between 5 and 0 days prior to D0. This initial rise is followed by a mean elevation of 10.1 ± 8.5 ng/mg CR/day from 1 through 17 days post-D0. Mean PDG levels are 125 ± 13.4 ng/mg CR (range 40.0–278.0 ng/mg CR) from 5 days prior through 18 days post-D0. The mean luteal phase, defined as the interval from D0 to the day of the precipitous decline of PDG, was determined to be 19.0 days (range 17-21).

The urinary ES and PDG excretion patterns during gestation in a single Indian rhinoceros are illustrated in Figure 3. Observed estrus and breeding occurred approximately 1 month prior to the first sample analyzed. ES concentrations throughout gestation are similiar to baseline levels (day 25 through 18 days prior to D0) in nonpregnant animals. PDG concentrations at 2 months postbreeding are within the range of luteal phase levels found during nonfertile cycles. By 3 months post breeding, PDG levels rise to above 1.0 µg/mg CR and continue to rise through the gestation period. Two weeks prior to parturition, levels rise sharply, to above 10.0 µg/mg CR, then decline to 5.8 µg/mg CR 5 days prior to parturition. These levels drop almost tenfold 1 day postpartum and are within the baseline range 2 weeks postpartum.

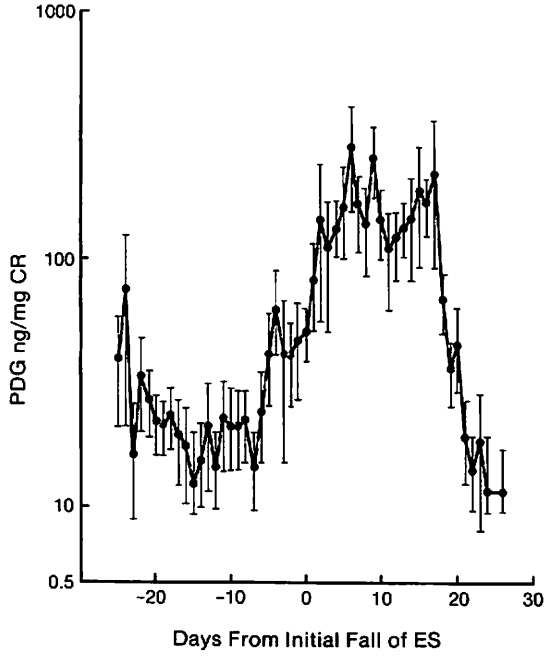

Fig. 2. Immunoreactive urinary pregnanediol-3-glucuronide (PDG) levels ($\bar{X} \pm$ SEM; n = 10) during the Indian rhinoceros estrous cycle. All values are aligned on the precipitous fall of estrone sulfate (ES) and are indexed by creatinine (CR).

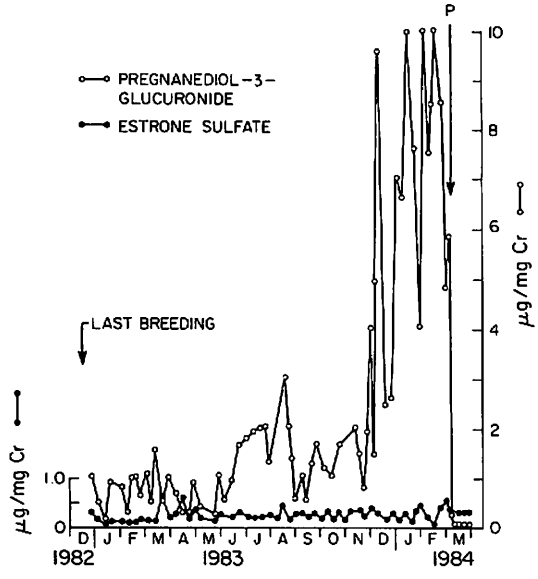

Fig. 3. Immunoreactive urinary levels of estrone sulfate and pregnanediol-3-glucuronide, indexed by creatinine (Cr), during gestation of an Indian rhinoceros.

DISCUSSION

The present data demonstrate that daily urine samples can be collected and analyzed to accurately reflect the major reproductive events in the female Indian rhinoceros. The ability to measure both urinary estrogen and progesterone metabolites provides for the assessment of follicular growth, ovulation, formation of an active corpus luteum, and determination of pregnancy within 3 months postconception. Taken together, the combined estrogen and progesterone metabolite profiles present a complete evaluation of ovarian steroid production in this species.

Each phase of the female Indian rhinoceros reproductive cycle can be characterized by discrete levels of urinary estrone sulfate (ES) or pregnanediol-3-glucuronide (PDG). The follicular phase is reflected by a gradual rise of ES, peaking for several days, then sharply declining, indicating the formation and regression of an ovarian follicle. The mean follicular phase length determined by this study is consistent with previous observations of 13–18 days based on total urinary estrogen excretion [Kassam and Lasley, 1981]. The rise of PDG at the time of declining ES reflects the formation of an active corpus luteum and confirms the complete ovulatory process. All of the nonpregnant animals in this study exhibited two repetitive ovulatory cycles, as indicated by a postestrus rise in PDG. Although no animals were bred during the study period, the temporal consistency of hormone profiles between individual profiles and among animals suggests that the present data represent normal ovulatory events.

The sustained elevation of PDG found in early pregnancy as compared to the clear biphasic shift of PDG levels in nonfertile cycles suggests that conception can be ascertained within 3 months after successful breeding. Additionally, the maintenance of urinary PDG above luteal phase levels throughout gestation provides the ability for the continous monitoring of gestation. The sharp decline of PDG found several days prior to parturition may provide for the accurate prediction of imminent delivery.

The source of the large range of hormone values reported for the five cycling animals is open to a number of possible explanations. Urinary hormone metabolites may reflect animal-to-animal variation as a result of differences in gut metabolism and resorption of steroid metabolites. Though creatinine determinations are used to compensate for the differences in daily fluid intake and output, this method was assumed to be of value but has not been rigorously verified in this species. More likely, the absolute hormonal variation between animals represents the true differences to be found in an heterogeneous, small population. This suggests that longitudinal sampling is essential to evaluate reproductive status of individuals.

The data presented in this report may be applied at several levels of captive rhinoceros breeding and management. Reproductive status can now be assessed prior to animal introductions and prior to interzoo movement of individuals. Urinary hormone analysis offers the zoo veterinarian a diagnostic approach to the infertile female rhinoceros, allowing for accurate diagnosis of ovarian dysfunction and application of appropriate therapy. Additionally, treatments relating to ovarian function can be monitored and modified according to response. The ability to diagnose pregnancy and ascertain if gestation is progressing normally can assist in making management decisions concerning individual females and may help assure neonatal survival. The utilization of these assays should also assist in the development of

advanced reproductive techniques for the Indian rhinoceros, such as estrous cycle synchronization and artificial insemination.

The ES and PDG data demonstrate that all aspects of Indian rhinoceros ovarian function can be evaluated. Utilizing this strategy, detailed information regarding the reproductive physiology of the Indian rhinoceros can be accumulated. The development of improved management and breeding techniques should be the direct result of a comprehensive understanding of the basic physiology of this species.

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