

Pregnancy Diagnosis in the Black Rhinoceros (*Diceros bicornis*) by Salivary Hormone Analysis

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Saliva samples collected from 12 black rhinoceroses (*Diceros bicornis*) were analyzed for 20 alpha-hydroxyprogesterone-like and total estrogen immunoreactivity. Five nonpregnant, two conception, and nine pregnancy intervals were monitored. HPLC co-chromatography of immunoreactive free steroid components in saliva indicated that one of three immunoreactive progestin components eluted with 20 alpha-hydroxyprog-4-en-3-one, whereas the single major estrogen peak eluted with estradiol. Direct radioimmunoassay measurements of ether extracts for these two reproductive hormones in saliva provided accurate and consistent profiles in which pregnancy was detected 13 months prior to parturition, and parturition was predicted by approximately 1 month. Measurement of 20 alpha-hydroxyprog-4-en-3-one was more useful in pregnancy diagnosis, and estradiol measurement was useful as an indicator of impending parturition.

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INTRODUCTION

The stability of the African black rhino (*Diceros bicornis*) population in the wild and in captivity is a matter of international concern. The numbers of the free-ranging population of this rhinoceros species have plummeted, dropping from an estimated 65,000 in 1970 to today's less than 4,000, due to severe poaching [Martin, 1993]. The captive population (210 animals), while secure from poaching, suffers from a low birthrate. While most captive mature female black rhinoceroses have reproduced in the past, approximately 85% have produced only one offspring while in captivity [Foose, 1994]. This suggests a possible unknown cause of subfertility in the captive population. In order to better understand normal ovarian and placental

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function, and to assess and treat specific infertility, it is essential to be able to measure reproductive hormones.

Previous studies of urinary steroid hormones in the Indian rhinoceros have proven practical for monitoring ovarian events and establishing the hormonal parameters of gestation [Kasman et al., 1986]. However, a similar approach has not been useful for assessing ovulation or diagnosing pregnancy during the first 7 months of the 15 month gestation period in the black rhino. In an effort to establish an alternative technology which does not require anesthesia or restraint in order to diagnose pregnancy, black rhino salivary hormone levels were investigated. Salivary hormone analysis has been applied extensively in humans as a means of evaluating endocrine function [Riad-Fahmy et al., 1982], to diagnose hormonal-related genetic disorders [Zerah et al., 1987], and to examine correlates with reproductive ecology [Ellison, 1988]. Recently, studies of salivary hormones have been extended to exotic species such as the macaque [Arslan et al., 1984] and the pygmy hippopotamus [Dathe and Kuckelhorn, 1989]. The present study was undertaken to determine if standard free steroid assays could be used to evaluate salivary samples as a means of diagnosing pregnancy and monitoring reproductive function in the black rhinoceros.

MATERIALS AND METHODS

Animals

Twelve sexually mature female black rhinoceroses monitored in this study were maintained at several zoological parks (Chicago-Brookfield, Chicago-Lincoln Park, Denver, Detroit, Los Angeles, Miami, St. Louis, San Antonio, San Diego). Five nonpregnant, two conception, and nine pregnancy intervals were monitored. Animals were maintained in both indoor and outdoor enclosures, given free access to water, and fed alfalfa, grass hay, fruits, and vegetables.

Salivation was stimulated with food. Foods such as bananas or carrots were used to increase salivation so that saliva pooled in the mouth. Saliva was collected without restraint of the animals, and at any time of day. Saliva samples were collected daily or on alternate days directly from the mouth using a feeding syringe with the nipple cut to allow for a wide opening. Samples were frozen (-20°C) as collected, without centrifugation until analysis.

Assays

One milliliter of saliva was mixed with tritiated estradiol (1,000 cpm, NEN Dupont, Boston, MA) to monitor for procedural losses during extraction. Saliva was extracted for 1 min with 5 ml freshly opened diethyl ether (Mallincrodt, Paris, KY). Following separation, the ether fraction was collected, dried, and reconstituted in 1 ml 0.1 M phosphate-buffered saline (pH 7.0). Equal aliquots (0.3 ml) were taken to count for procedural losses, to analyze for 20 alpha-hydroxypreg-4-en-3-one (20A-OH-P), and to analyze for estrogen (E). Radioimmunoassay for total estrogens [Hodges et al., 1979] was used to quantitate the estrogen content of saliva samples. The estrogen antiserum used in the total estrogen assay (Abraham S310 #5) cross-reacts with estriol 100%, estradiol- 17β 140%, and with estrone 90% [Czekala et al., 1983].

Free progesterone was measured from saliva and gave results which suggested that this steroid would not be a good monitor of pregnancy. As a result, 20A-OH-P,

a known major metabolite of progesterone in the white rhinoceros (*Ceratotherium simum simum*) [Hindle and Hodges, 1990], was examined as a potential progesterone metabolite in saliva for monitoring of pregnancy. Progestin content was therefore monitored using radioimmunoassay for 20A-OH-P. The antibody used in this assay crossreacts with 20A-OH-P 100%, pregnanediol-3-glucuronide (PDG) 53%, allo-pregnanediol 28%, and pregnanediol 16% [Monfort et al., 1989]. Interassay coefficient of variation was 11.4% (40% binding) and 25.6% (58% binding) for the estrogen and 20A-OH-P assays, respectively. Serial dilutions of pregnant black rhino saliva yielded a parallel dose-response curve to the specific standards for both 20A-OH-P ($r = 0.992$) and total estrogens ($r = 0.98$).

HPLC

Cochromatography was performed on the HPLC to monitor for both immunoreactivity and added radiolabel for the 20A-OH-P and total estrogen assays, to determine both the number and polarities of immunoreactive substances contributing to each assay (Figs. 1, 2). A salivary sample collected during the estrous period was spiked with radiolabeled estradiol and estrone, and extracted with diethyl ether, dried, and reconstituted in methanol. This prepared sample was injected into a Licrosorb (reverse phase, 10 μ) column at a flow rate of 1.0 ml/min using an isocratic acetonitrile:water (40:60) solvent. Effluent samples were collected in 0.3 ml/tube, and each fraction was tested for both immunoreactivity and radiolabel. A saliva sample collected during pregnancy was spiked with radiolabeled 20A-OH-P and pregnanediol-3-glucuronide. HPLC separation of progestins from diethyl ether-extracted pregnancy saliva used a Licrosorb column and an acetonitrile:water gradient as follows: 0–10 min 40% acetonitrile; 10–20 min with a gradient of 40–100% acetonitrile; 20–25 min 100% acetonitrile (1 ml/min, 0.3 ml/tube). Radiolabeled markers were used to identify column characteristics while radioimmunoassay was used to identify crossreacting compounds.

Statistics

Mean concentrations of 20A-OH-P and E from each individual animal were calculated for each month of pregnancy or nonpregnancy. Nonpregnant animals were not selected from known normally cycling females, nor were samples selected from a particular phase of the cycle. Group *t* test analysis was applied to determine the significant difference between nonpregnant hormone concentrations and concentrations of hormone during the different months of pregnancy.

RESULTS

Salivary progestins using 20A-OH-P radioimmunoassay

The HPLC separation of progestins using 20A-OH-P radioimmunoassay of a saliva sample taken during pregnancy is shown in Figure 1. In tube 70 an immunoreactive peak coeluted with the 20A-OH-P radioactive label, suggesting that this progestin is present in pregnant black rhino saliva. In tube 10, another smaller peak coeluted with the PDG label. The major immunoreactive peak in tube 73 was not identified.

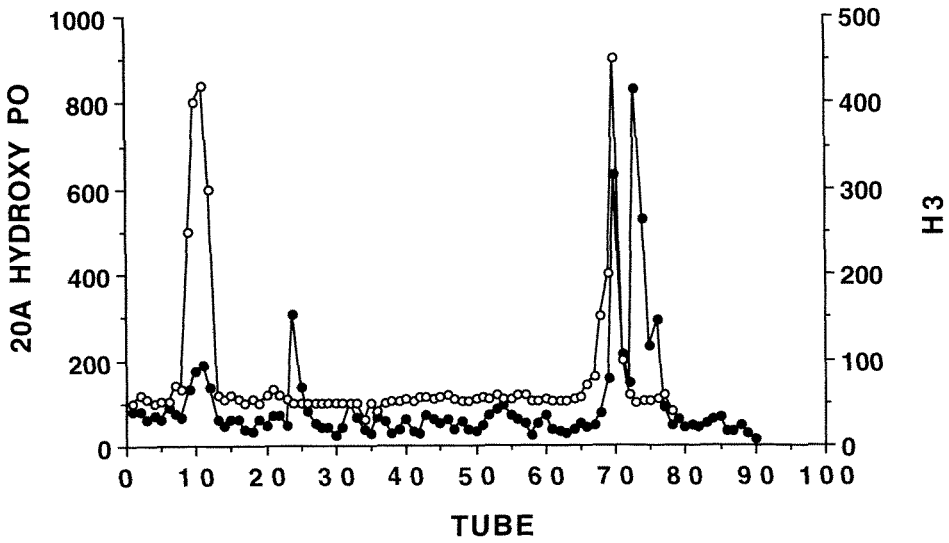


Fig. 1. Cochromatography of pregnancy saliva using HPLC and radioimmunoassay of 20 alpha-hydroxyprogesterone. Closed circles represent immunoreactive compounds and open circles represent radiolabeled compounds. Radiolabel eluting at tube 10 is PDG and at tube 70 is 20A-OH-P.

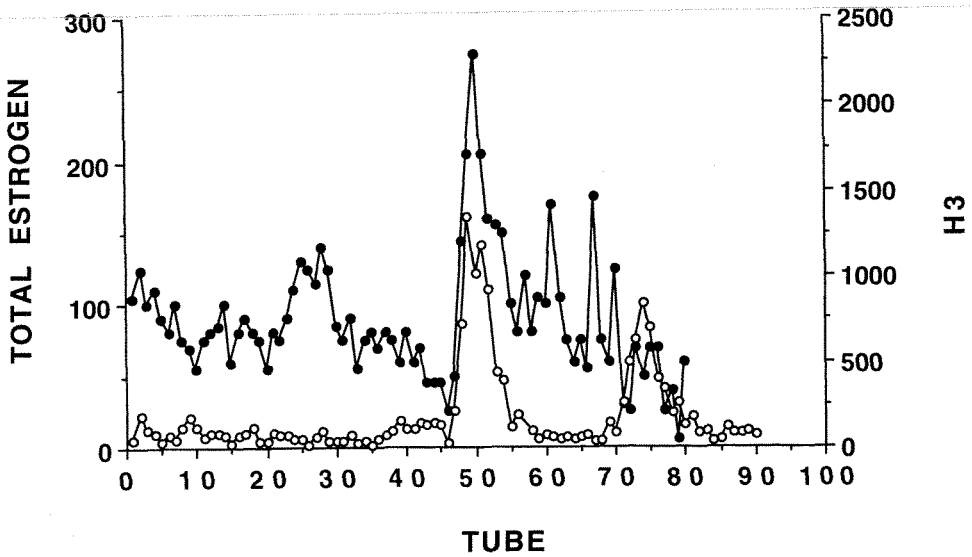


Fig. 2. Cochromatography of estrus saliva, using HPLC and radioimmunoassay of total estrogen. Closed circles represent immunoreactive compounds and open circles represent radiolabeled compounds. Radiolabel eluting at tubes 49-50 represents estradiol, and at tube 74, estrone.

Salivary estrogens using total estrogen radioimmunoassay

Figure 2 illustrates the HPLC separation of estrogens from saliva collected near the time of estrus. Two significant immunoreactive peaks were observed. The major



Fig. 3. Mean \pm SEM concentrations of 20 alpha-hydroxyprogesterone in saliva during normal gestation. Asterisks indicate P values < 0.05 when compared to nonpregnant concentrations. Numbers within bars indicate the number of individual rhinos used at each month of gestation.

peak in tube 50 coeluted with radiolabeled estradiol-17 β . The smaller immunoreactive peak in tube 28 was not identified. No immunoreactive peak coeluting with the estrone radiolabel marker was observed.

A comparison of salivary 20A-OH-P concentrations from both nonpregnant animals and animals sampled during gestation (Fig. 3) indicated a general elevation during early pregnancy (month -15). When compared to nonpregnant levels, these early elevated concentrations did not become statistically significant until 13 months before delivery. The rise in salivary concentrations of 20A-OH-P plateaued at 11 months before delivery and remained elevated until parturition. Salivary estrogens from pregnant animals (Fig. 4) also showed a similar early rise (months 14 and 13 before delivery), followed by a decrease to concentrations significantly below those found in nonpregnant animals (months 10 and 8 before delivery; $P = 0.04$ and 0.032 , respectively). Concentrations of estrogen then increased again as the time of parturition approached. The mean concentration of estrogen at 1 month prior to delivery was elevated significantly ($P = 0.0059$) above concentrations found in nonpregnant animals. Using these parameters, no false positive or negative pregnancy determinations have occurred.

DISCUSSION

The results from HPLC analysis suggest that both salivary 20A-OH-P and estradiol are present and detected by the assays used for determining pregnancy and monitoring its progress in the black rhinoceros. Although the origin and metabolism of 20A-OH-P has not been examined in the black rhino, Hindel and Hodges [1990] found that progesterone was converted into 20A-OH-P in the related white rhinoceros. The observed increase in salivary 20A-OH-P which began 15 months prior to

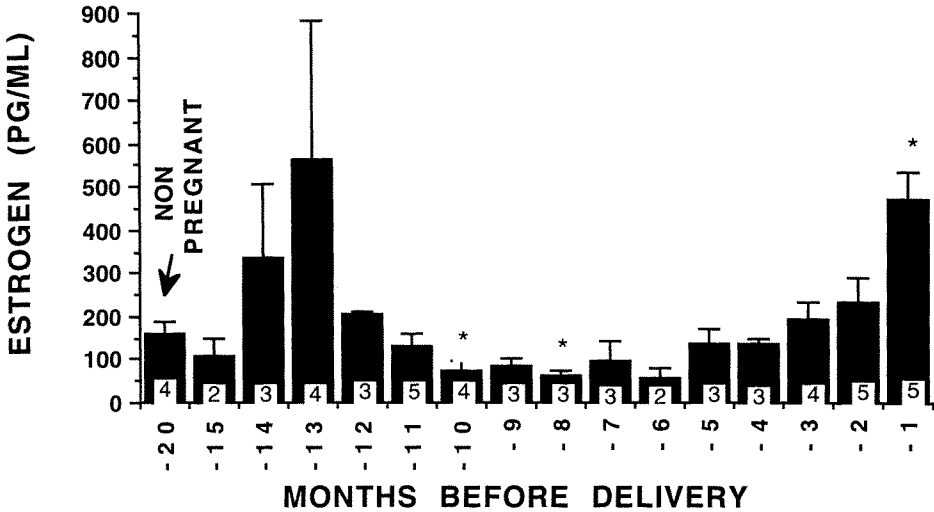


Fig. 4. Mean \pm SEM concentrations of total estrogen in saliva during normal gestation. Asterisks indicate $P < 0.05$ when compared to nonpregnant concentrations. Numbers within bars indicate the number of individual rhinos used at each month of gestation.

parturition and plateaued at 11 months from parturition until delivery is similar to that of one of its metabolites, PDG, found in the urine of the pregnant Indian rhinoceros [Kasman et al., 1986]. In the Kasman study, urinary PDG concentrations remained at luteal phase levels until 10 months before delivery, then rose to plateau at a higher concentration until parturition. In the Indian rhino a sharp increase in urinary PDG occurred 2 weeks prior to parturition. This increase was not observed in the black rhino salivary hormones, as shown in Figure 3. Results from a urinary hormone study in the pregnant black rhino did not reveal any PDG elevation until 7 months before delivery [Ramsay et al., 1987]. However, the results from a fecal hormone study in the pregnant black rhino measuring progesterone 20 alpha-hydroxyl or 20-keto metabolites [Schwarzenberger et al., 1993] show a hormonal pattern during pregnancy similar to ours for 20A-OH-P salivary hormones. The similarities in the antisera crossreactivities in these two studies suggest that these metabolites are important monitors of pregnancy in the black rhino.

The increase in black rhino salivary estrogens measured during early pregnancy was not significantly elevated above concentrations found in nonpregnant animals (Fig. 4). Salivary estrogen concentrations were distributed widely during the first 4 months of pregnancy. At 10 and 8 months prior to delivery, the concentrations were significantly lower than nonpregnant concentrations. Estrogen concentrations remained near baseline until 5 months before delivery, and then underwent a gradual but significant increase until 1 month before delivery. This pattern was not observed in the analysis of urinary estrogens from a variety of perissodactyla species. In the Indian rhinoceros, urinary estrogen levels remained close to baseline throughout gestation [Kasman et al., 1986]. In the tapir, urinary estrogens rise above baseline approximately 7 months prior to delivery [Kasman et al., 1985]. The level of urinary estrone conjugates found in the pregnant black rhinoceros was not detectably different

from those of nonpregnant females [Ramsay et al., 1987]. In contrast, urinary estrone sulfate levels in the domestic horse rose significantly after 45 days postconception [Evans et al., 1984]. The black rhino salivary estrogen increase at 14 and 13 months prior to parturition, while not a statistically significant increase, may indicate a role of chorionic gonadotropin in early pregnancy similar to that found in the horse.

The salivary steroids estradiol and 20A-OH-P are reliable markers for pregnancy detection in the black rhinoceros. Using analysis of 20A-OH-P from a single sample, pregnancy can be diagnosed as early as 13 months before delivery. Salivary estrogen is more informative as a pregnancy detection tool used in combination with 20A-OH-P results and as a means of predicting impending parturition.

CONCLUSIONS

1. Measurement of salivary 20A-OH-P can detect pregnancy as early as 13 months before delivery in the black rhino.
2. Measurement of salivary estradiol in concert with measurement of 20A-OH-P can be used to follow the progress of pregnancy.
3. Salivary estradiol increases significantly during the month prior to parturition.

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