PREGNANCY DETERMINATION IN UNCAPTURED FERAL HORSES BY MEANS OF FECAL STEROID CONJUGATES

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ABSTRACT

This study was carried out to develop an accurate, rapid and inexpensive method for diagnosing pregnancy in uncaptured feral horses by analysis of fecal steroid metabolites and to compare the accuracy of this method with diagnosis by urinary estrone conjugates (E_1C). Paired urine and fecal samples were collected from 40 sexually mature feral mares during August and October. Urine samples were extracted directly from the soil and analyzed by enzymeimmunoassay (EIA) for E_1C. Water extracts of fecal samples were assayed by EIA for E_1C and nonspecific progesterone metabolites (iPdG). Urinary E_1C, fecal E_1C and fecal iPdG concentrations for seven mares which produced foals were 3.9 ± 1.3 (SEM) µg/mg creatinine, 4.2 ± 0.8 ng/g feces and 1,411 ± 569.6 ng/g feces, respectively. Urinary E_1C and fecal E_1C and iPdG concentrations for the 33 mares which did not produce foals were 0.1 ± 0.0 µg/mg creatinine and 0.5 ± 0.1 and 32.8 ± 4.5 ng/g feces, respectively. These differed ($P < 0.01$) from values in mares which produced foals.

Key words: pregnancy, horse, fecal steroids, progestins, estrogens

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INTRODUCTION

Pregnancy determination in uncaptured feral horses has been accomplished by analysis of urinary estrone conjugates (E_1C;1,2), urinary pregnanediol-3∂-glucuronide-like progesterone metabolites (iPdG), and fecal total estrogens (3-5). The ability to diagnose pregnancy in free-roaming ungulates is important to understand environmental influences on reproductive parameters such as fetal loss. Immobilization and capture are necessary to diagnose pregnancy by means of standard immunological tests or with plasma hormone concentrations, but capture events are associated with enormous physical stress for the animal, significant rates of mortality (6,7) and disruption of reproductive events (8). The field collection of urine from free-ranging feral horses, however, is a time-consuming process and, while radioimmunoassay of free total fecal estrogens is an accurate means of diagnosing pregnancy, not all wildlife biologists have access to laboratory facilities for isotopic assays. The ideal approach to pregnancy diagnosis in feral horses would utilize a rapid, inexpensive and accurate nonisotopic assay of some steroid or its metabolite in fecal samples. The present study was carried out to develop such a method for diagnosing pregnancy in feral horses by enzymeimmunoassay of fecal steroid metabolites and to compare this method to the urinary E_1C method.

MATERIALS AND METHODS

Paired urine and fecal samples were collected from 40 sexually mature free-roaming feral mares inhabiting Assateague Island National Seashore (Maryland), an Atlantic coast barrier island. These mares are part of a free-roaming herd of approximately 150 feral horses which have been intensively studied since 1975. The parentage, ages, fertility, band affiliations and band movements have been recorded for more than 90% of the entire herd. The mares chosen for the study possessed unique identifying markings and were photographed for later identification. Urine and fecal collections were carried out during August and October 1989. The horses were observed from a distance of 30-50 m, and urine was collected by direct aspiration from the ground as described by Kirkpatrick et al. (1) or by hand-centrifugation from beach sand; urine samples were placed on ice immediately after collection and stored frozen without a preservative. Fecal samples were placed in a plastic self-sealing bag and stored frozen.

Unextracted urine samples were analyzed for estrone conjugates (both estrone sulfate and estrone glucuronide) by enzymeimmunoassay (EIA) as described previously (3,9). Urinary creatinine (Cr) was analyzed by the microcolorimetric method of Taussky (10) and E_1C values were reported as µg/mg Cr in order to account for differences in urine concentration. Approximately 0.5 g of feces was suspended in 5.0 ml of distilled H_2O (dH_2O), shaken for hr and centrifuged for 10 minutes at 2500 rpm in a refrigerated centrifuge. Twenty microliters of the recovered supernatant were assayed for E_1C concentration by EIA as previously described (3) and for non-specific progesterone metabolites (iPdG) by EIA using an antibody raised against PdG, but with cross-reactivity to 20∂-hydroxyprogesterone (11). Fecal E_1C and iPdG values were reported as ng/g feces. Sensitivity of the assay, based on the lowest value for measureable PdG standards in distilled H_2O, which differed from

the blank value ($P<0.05$) was between 40 and 70 pg/well. The inter- and intraassay coefficients of variation, based on the measurement of internal standards, were 14% (n=5) and 9% (n=5) respectively. No corrections were made for the water content of the feces. Approximately 5000 cpm of ^{3}H-estrone sulfate (E_1S) was added to 10 separate 0.5-g wet fecal samples. These samples were treated as described above, the supernatant was evaporated and the residue counted for activity in to assess recovery. Recovery experiments could not be conducted fer iPdG as the precise nature of the metabolites is not known. Consequently, iPdG values are estimates which are used for pregnancy diagnosis and not absolute values.

To validate the fecal E_1C and iPdG assay for the mare, a water extract from a pregnant mare fecal sample was subjected to high performance liquid co-chromatography (HPLC) as described by Kirkpatrick et al. (11). The fecal sample was prepared as described above and 1.0 ml of the supernatant was extracted twice with 3.5 ml of diethyl ether to remove free sterolds. One milliliter of methanol:ethanol (1:1) was added to the fecal supernatant, and the mixture was vortexed, cooled to -5^{O} C and centrifuged at 2500 rpm for 10 min to remove salts. Approximately 1000 cpm of ^{3}H-estrone sulfate (estrone sulfate, ammonium salt (6,7-3H(N)), New England Nuclear) or ^{3}H-PdG (20-40 Cl/mmole;Courtald Institute, London) were added to the supernatant, which was reduced to approximately 1.0 ml volume under N_2. Twenty-five microliters were injected onto a 15-cm HPLC column (ALTEX, Ultrasphere-ODS, dp 5 µ). One milliliter fractions were collected during a MeOH/H2O gradient (10 to 65% MeOH over 40 min.). From each fraction, 200 µl were mixed with 5.0 ml of scintillation cocktail (Ready Protein, Beckman) to monitor tritiated E_1S or PdG recovery. The remaining 800 µl were evaporated, and the residue was dissolved in 100 µl of dH_2O and assayed for E_1S or iPdG as described above. Reliability characteristics for the E_1C and iPdG EIAs have been previously reported (3,9,11). The mares were relocated during the summer of 1990 and observed for the presence of foals. Differences in the mean concentrations of hormone metabolites were tested for significance with standard t-tests.

RESULTS

Seven of the 40 mares produced foals and had urinary E_1C concentrations ranging from 1.3 to 10.3 µg/mg Cr, with a mean of 3.9 ± 1.3 (SEM). The 33 mares which did not produce foals had urinary E_1C concentrations ranging from nondetectable to 0.6 µg/mg Cr, with a mean of 0.1 ± 0.0. Based on the presence of foals in 1990 and a cut-off of 0.7 µg/mg Cr, prediction of foal production was 100 % accurate based on urinary E_1C concentrations. Fecal E_1C concentrations for the seven mares which produced foals ranged from 1.7 to 6.1 ng/g feces, with a mean of 4.2 ± 0.8. Fecal iPdG ranged from 217.5 to 4,359.5 ng/g, with a mean of 1,411.5 ± 569.6. The 33 mares which did not produce foals had fecal E_1C concentrations ranging from 0.2 to 1.5 ng/g feces, with a mean of 0.5 ± 0.1. Fecal iPdG concentrations in those mares ranged from 1.2 to 142.6 ng/g, with a mean of 32.8 ± 4.5. Differences between the means of urinary E_1C and fecal E_1C and iPdG for mares which produced foals and those which did not were significant at the $P < 0.01$ level of confidence. Using 150 ng fecal iPdG/g feces as a predictor of foal

production, this assay was also 100 % accurate. Fecal E_1C, using 1.0 ng/g feces as a predictor of foal production, was 95% accurate. The results are summarized below (Table 1).

Table I. Fecal E_1C and iPdG concentrations for pregnant and non pregnant mares (± SEM)

Mare status	N	Urinary E_1C (µg/mg Cr)	Fecal E_1C (ng/g)	Fecal iPdG (ng/g)
Pregnant	7	3.9±1.3	4.2±0.8	1,411±569
Non-pregnant	33	0.1±0.0	0.5±0.1	32.8±4.5

Recovery of ^{3}H-E_1S from fecal samples was 87 ± 6.7 %. Enzymeimmunoassay analysis of HPLC separated fractions of fecal supernatants spiked with ^{3}H-estrone sulfate revealed consistency between the fractions with label and those with immunoreactivity to the E_1C antibody, thus indicating that E_1C is the major metabolite in equine fecal samples measured by the EIA (Figure 1). Enzymeimmunoassay analysis of HPLC - separated fractions of fecal supernatants spiked with ^{3}H-PdG, however, revealed the presence of several major peaks, which demonstrated immunoreactivity with the iPdG antibody, three of which preceded the eluates containing ^{3}H-PdG (Figure 2). The HPLC co-chromatography profile of fecal iPdG is consistent with the pattern seen in urine (11).

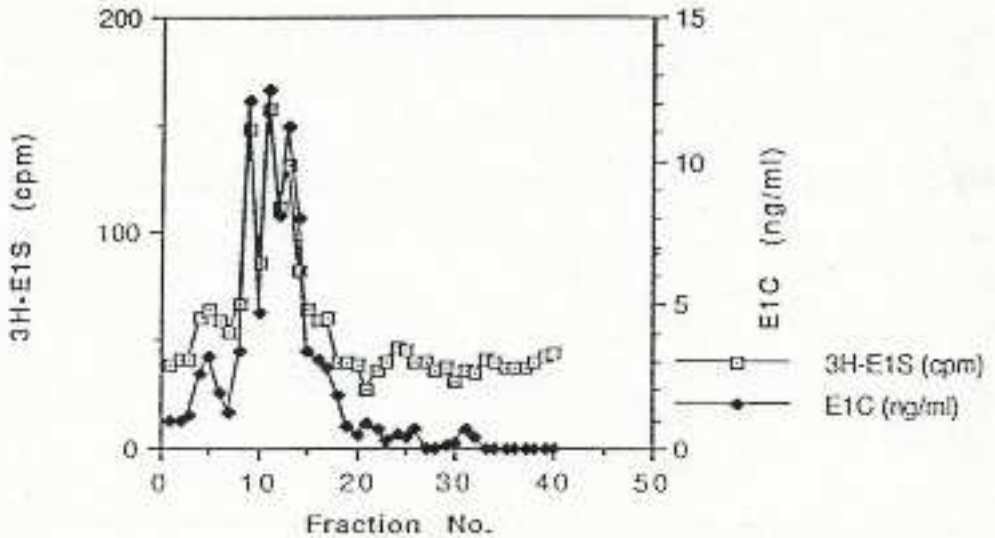

Figure 1. An HPLC elution profile of ^{3}H-estrone sulfate and immunoreactive estrone conjugates, including estrone sulfate and estrone glucuronide.

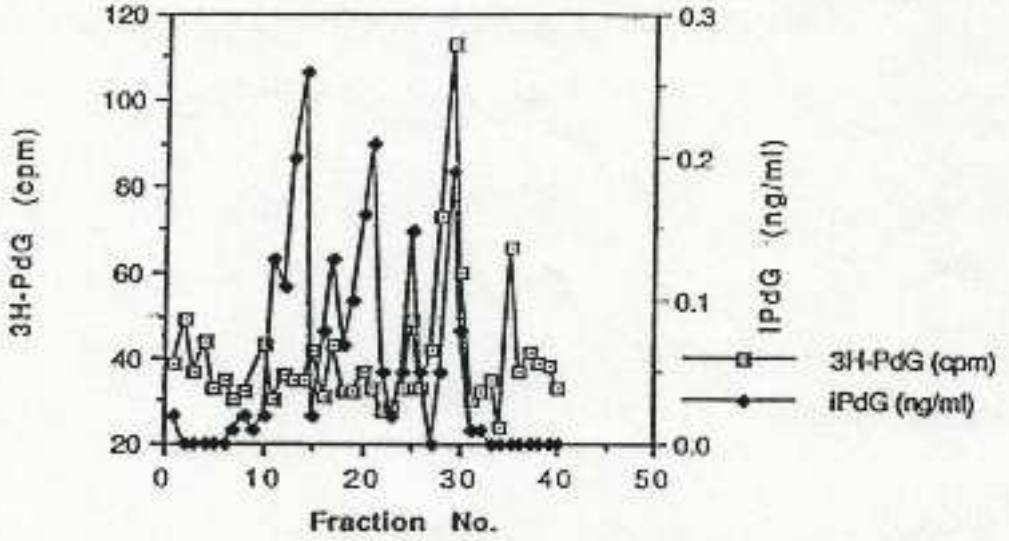

Figure 2. HPLC elution profile of ^{3}H-PdG and immunoreactive non-specific progesterone metabolites (iPdG).

DISCUSSION

The assay of aqueous extracts of feces using the EIA for E_1C and the EIA for iPdG provides an accurate predictor of foal production in feral mares. Both of these steroid conjugates have been previously identified in the urine of pregnant mares (11,12), which is thought to be the main route of their excretion. Despite the extensive resorption of conjugated steroids from the gastrointestinal tract and their primary clearance through the kidney, it was reasonable to suspect that small but significant quantities of these steroid metabolites were excreted in the feces. Sist et al. (13) have identified the presence of estrone sulfate (E_1S) in mare feces, and HPLC supports the presence of E_1S in the immunoreactive fractions of mare feces in this study. The HPLC profile of aqueous fecal fractions which were immunoreactive with the nonspecific iPdG antibody is similar to that found in mare urine (11), with three major peaks eluting earlier than PdG. These data suggest that the immunoreactive steroid metabolites of feces in the mare are the same as those previously reported in urine. Although the identity of the progesterone metabolites found in urine of nonpregnant mares is not yet known, their origin as ovarian progesterone has previously been demonstrated in cyclic mares (11).

In field studies of this nature, it is not possible to confirm pregnancy by traditional invasive methods among the mares at the time of urine or fecal collection, thus the accuracy of actual pregnancy detection at the time of collection is only implied among feral mares in this study. However, the high degree of accuracy of pregnancy detection by means of urinary E_1C concentrations has been previously tested in domestic mares and confirmed by rectal palpation (12,14). More recently, this accuracy has been confirmed in other species of the Equidae, including zebras and Przewalski's horses (15,16).

The use of either the fecal E_1C or iPdG analysis alone for pregnancy determination poses two minor risks and the possibility of error. In a previous

study (2), October urinary E_1C determinations in 34 mares proved to be 100 % accurate in predicting foals, but in the present study two mares which did not produce foals had E_1C concentrations in excess of 1.0 µg/mg Cr. One of those two mares was witnessed displaying clitoral winking and was successfully mounted by a stallion during the October collection period. This behavior is characteristic of nonpregnancy and suggests nonovulatory folliculogenesis, a phenomenon reported in western feral mares(17), in which plasma estrogens occasionally and abruptly rise during the seasonally anestrus fall months but without the occurrence of ovulation. Thus, an occasional nonpregnant mare may confound a diagnosis based on urinary E_1C alone. Although the iPdG measurements produced 100% accuracy in predicting foals in the present study, it is possible that mares with persistent corpora lutea could produce urinary iPdG values indicative of pregnancy. Cyclic mares have been shown to excrete concentrations of urinary iPdG in the 250 to 400 ng/mg Cr range (11), but the range for fecal iPdG in the cyclic mare is not yet known. For the greatest accuracy, both fecal assays should be employed.

Since fecal iPdG concentrations have not yet been evaluated in cyclic mares, there is no way of knowing whether values found in cyclic mares would interfere with pregnancy diagnosis. On Assateague Island, 88% of the mares cease to cycle after July. Foaling records over an eight-year period indicate that less than 10% of foals are born after July (18). With 88% of matings having occurred by the end of July, most pregnancies in the present study presumably were at least 35 d post conception by the earliest collection date in late August. Thus the probability that urine/fecal collections occurred within the post-conception period in which urinary E_1C concentrations began to exceed 1.0 µg/mg Cr is high. Among feral horses estrus and ovulation are exceedingly rare after August, thus October fecal collections minimize the chances of confusing pregnancy with estrous cycles in tested mares.

The implications of successful pregnancy diagnosis by means of fecal steroid metabolites in horses and other large mammals are important to field biologists. Ovarian function and pregnancy have been monitored in a large number of captive exotic ungulate species by means of urinary steroid metabolites (20), and it is likely that pregnancy can be determined in these same species by means of fecal steroid metabolites. The ease with which fecal samples can be collected provides a clear advantage over urine collection when working with secretive animals like deer, or dangerous species such as the bison. The nonisotopic nature of the enzymeimmunoassay eliminates the need for the sensitive gamma and beta detectors required for radioimmunoassay and makes these tests relatively simple and inexpensive.

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