DEVELOPMENT AND UTILITY OF FECAL PROGESTERONE ANALYSIS TO ASSESS REPORDUCTIVE STATUS IN FELIDS

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Understanding basic reproductive processes is crucial for determining why some animals reproduce and others do not. Some nondomestic felid species reproduce poorly in captivity, and there is little physiological information available to help identify what causes reproductive failure in certain species, populations or individuals. One of our goals is to develop databases on the hormonal interrelationships regulating reproductive activity. However, monitoring hormonal patterns using traditional blood sampling methods is not practical for these species. Instead, noninvasive techniques for assessing reproductive function need to be developed. Metabolism of estradiol (E_2) has been described for the domestic cat, and it was found that >90% of this steroid was excreted in feces which explains the failure of urinary monitoring techniques to track reproductive patterns in felids.[1,2] Our objectives were to determine excretion rates and metabolism of progesterone (P_4) in the domestic cat, and then to develop and validate a fecal progestogen radioimmunoassay (RIA) for monitoring ovarian function in the domestic cat and representative nondomestic felid species.

A radiolabel infusion study was conducted in the domestic cat to assess P_4 metabolism and excretion. Three anestrous females were injected i.m. with 3 *u*Ci ^{14}C-P_4 (¯56 mCi/mmol; New England Nuclear) and 100 *u*g unlabeled P_4 in 2.0 ml 0.9% NaCl. Following isotope injection, syringes were rinsed with ethanol and the residual radioactivity counted and subtracted from the pre-injection total. Cages were checked at ~2 h intervals and all urine and feces collected for 5 d post-injection. Feces and urine contained 96.7 $\pm$ 0.5 and 2.6 $\pm$ 1.2%, respectively, of the radioactivity as a percentage of total radioactivity administered. The majority of radioactivity in the urine was detected in the first sample collected between 8 and 13 h post-injection (11 $\pm$ 1 h). Peak radioactivity in feces also occurred in the first sample collected between 11 and 50 h (28 $\pm$ 11 h). Based on differential extraction with diethyl ether (1:10 v/v), 78% of P_4 metabolites were excreted as conjugated (aqueous phase) rather than free (organic phase) steroid forms. Using a gradient of 30-100% acetonitrile:dH_2O over 80 min (1 ml fractions, 1 ml/min flow rate), HPLC analyses detected 3 radioactive peaks, 2 polar (presumably conjugate) peaks at fractions 5-7 and 8-10 (48 and 42% of the total radioactivity, respectively) and a less polar peak at fractions 38-39 (10%). None of these peaks co-eluted with the ^{3}H-P_4 reference tracer (fractions 35-36). Conjugated metabolites were not enzyme-hydrolyzable, and, based on HPLC analyses, the percent hydrolyzable by sulphatase (8%) and B-gkycyribudase (7%) appeared to represent residual conjugates entering the ether phase.

A rapid method for extracting fecal P_4 metabolites (herein referred to as progestogens) from cat feces was developed based on the method of Wasser *et al.*[3] Dried, pulverized fecal material (0.1-0.2 g) was boiled in 5 ml of 88% ethanol:dH_2O for 20 min and centrifuged at 500 *g* for 10 min. the supernatant was recovered and the pellet resuspended in 5 ml of 88% ethanol, vortexed for 1 min and recentrifuged. The two ethanol phase supernatants were combined, dried completely and then redissolved in 1 ml methanol. This technique resulted in a recovery of 87.2 $\pm$ 1.4% (n=6) for metabolized ^{14}C-P_4, and > 90% for exogenous ^{14}C-P_4 added to fecal samples before extraction. The P_4 RIA developed in this laboratory utilized a monoclonal P_4 antibody (produced against 4-P-11-ol-3, 20-dione hemisuccinate:BSA) provided by Dr. Jan Roser (University of California, Davis), an ^{125}I-labeled P_4 tracer (Pantex, Santa Monica, CA) and P_4 standards.

The assay was incubated at 4°C for 24 h in a total volume of 500 *u*l. Standards (100*u*l) and/or sample (diluted 1:800 to 1:8,000 with RIA buffer; 0.01 M PO_4, 0.5% BSA, 2mM EDTA, 0.9% NaCl, 0.01% thimerolal, pH 7.4) were incubated with antibody (1:100,000 in 100 *u*l and P_4 tracer (20,000 c.p.m. in 100 *u*l) for 4 h. antibody-bound complexes were precipitated with sheep anti-mouse gamma globulin (1:80 in 200 *u*l; 20 h incubation) followed by addition of 1 ml rinse buffer (0.01 M PO_4 , 0.9% NaCl, 0.01% thimerosal, 5% polyethylene glycol, pH 7.4) and centrifugation for 30 min at 1500 *g*. The antibody bound 30-40% of the iodinated P_4 tracer with 3% nonspecific binding. Assay sensitivity, based on 90% of maximum binding, was 3 pg/ml, and the intra- and inter-assay coefficients of variation were <10%. This assay was validated for fecal extracts from the domestic cat *(Felis catus)*, leopard cat *(Felis bengalensis),* cheetah *(Acinonyx jubatus)* and clouded leopard *(Neofelis nebulosa)* by demonstrating: 1) parallelism between dilutions of pooled fecal extracts and the standard curve; and 2) significant recovery of exogenous P_4 (3.75-120 pg) added to fecal extracts (domestic cat, $y = 0.95x + 1.11$; $r = 0.99$; leopard cat, $y = 1.08x$-1.13, $r = 0.99$; cheetah, $y = 1.16x$- 0.42; $r = 0.99$; clouded leopard, $y = 1.08x$- 1.94; $r = 0.99$).

Co-chromatographic HPLC profiles of extracted fecal samples from the domestic cat revealed P_4 immunoreactivity coincided with 1 presumably conjugated (fractions 8-10) and 1 unconjugated (fractions 38-39) metabolized progestogen peak. Isotope infusions were not performed in nondomestic species, but progestogen immunoreactivity in leopard cat and clouded leopard fecal eluates was similarly associated with a conjugated (fractions 7-8; >90% of the total immunoreactivity) and unconjugated (fractions 38-39) peak, suggesting that P_4 metabolism may be similar among these species. Interestingly, RIA of fecal eluates from the cheetah revealed 3 immunoreactive peaks constituting 42% (fractions 7-8), 51% (fractions 43-36) and 7% (fractions 38-39) of the total radioactivity, with the second peak co-eluting with the ^{3}H-P_4 tracer. Thus, with the exception of the cheetah, fecal metabolites other than unconjugated P_4 appear to predominate in felids.

Longitudinal profiles of fecal progestogen excretion were examined in samples collected 3-5 times weekly for 2 wk before artificial insemination (AI) or natural mating through parturition to 2 wk after birth. Progestogen profiles were generated for 3 pregnancies resulting from AI (leopard cat, n = 1; clouded leopard, n=1) or natural mating (clouded leopard, n = 1), and for 5 nonpregnant luteal phases (i.e., pseudopregnancy) after AI (clouded leopard, n = 3) or natural mating (cheetah, n = 1; clouded leopard, n = 1). For AI, follicular development was stimulated using equine chorionic gonadotropin and ovulation was induced using human chorionic gonadotropin administered 5 and 2 d before laparoscopic AI, respectively. In the leopard cat, fecal progestogen concentrations were low before AI (5.2 $\pm$ 0.9 *u*g/g dry feces), increased within 5 d after AI and peaked at ¯1500 *u*g/g on Day 25 of pregnancy. Progestogen concentrations remained elevated throughout pregnancy (overall mean, 874 $\pm$ 66 *u*g/g); however levels gradually declined from ¯750 *u*g/g on Day 40 to ¯400 *u*g/g one day before parturition. Baseline concentrations were detected within ˜2 wk post-partum. Overall mean baseline progestogen concentrations in the clouded leopard (3.8 $\pm$ 1.7 *u*g/g) were similar (P> 0.05) to those observed in the leopard cat, whereas average basal concentrations in the cheetah (0.8 $\pm$ 0.2 *u*g/g) were lower (P< 0.05). Mean progestogen concentrations during the nonpregnant luteal phase (cheetah, 98.2 $\pm$ 10.3 *u*g/g; clouded leopard, 68.3 $\pm$ 8.1 *u*g/g) or pregnancy (clouded leopard, 41.1 $\pm$ 5.6 *u*g/g) were substantially lower (P< 0.05) than those observed in the leopard cat, suggesting that species differences exist in the quantity of progestogens excreted. Duration of pregnancy was 63 d in the leopard cat, and 88 and 89 d for the clouded leopard. The nonpregnant luteal phase duration was 50 d for the cheetah, and 45 $\pm$ 2.5 d (range, 39-51 d) for the clouded leopard, or about half that of a normal pregnancy.

In summary, P_4 in the domestic cat is metabolized and excreted primarily into feces. We have identified at least 3 major radioactive metabolite peaks using HPLC, although it is possible that several metabolites could be associated with each of these peaks. We also have developed and validated a P_4 RIA using an antibody that crossreacts with 2 of the 3 radioactive metabolite peaks present in the domestic cat. Although radiolabel infusion studies were not conducted in the nondomestic species, comparisons with the domestic cat suggest that the leopard cat and clouded leopard excrete similar immunoreactive metabolites, whereas the cheetah excretes an additional metabolite that may be unconjugated P_4 . These preliminary data provide good

evidence that fecal progestogen analyses are useful for monitoring changes in corpus luteum function during pregnancy and nonpregnancy in several felid species. We conclude that longitudinal monitoring of fecal progestogen excretion, in conjunction with fecal E_2 should provide a valuable tool for assessing ovarian function in felids. Given that this technology can be applied across taxa, then we predict that fecal steroid analyses will be valuable for: 1) characterizing ovarian activity over time; 2) determining the prevalence of induced versus spontaneous ovulation; and 3) providing valuable feedback on how to improve assisted reproduction strategies, making them a practical management tool. Because the duration of a nonpregnant luteal phase appears to be about half that of pregnancy, it may also be possible to distinguish between these two reproductive states during the second half of a suspected pregnancy.

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