J Vet Clin 27(6) : 693-697 (2010)

Journal of Veterinary Clinics
www.ksvc.or.kr

Time-resolved Fluoroimmunoassay (TR-FIA) Analysis of Fecal Progesterone and Estradiol in Leopard Cats (*Prionailurus bengalensis*)

Young-Seob Kim*, Ji-Yong Kim, So-Young Jung*, Bong-Joo Lee***[1] and Nam-Shik Shin**[1]**

**Seoul Zoo, Gwacheon, Gyeonggi-do 427-080, Korea*
***College of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea*
****College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, Korea*

(Accepted: December 10, 2010)

Abstract : This study, conducted with four leopard cats (*Prionailurus bengalensis*), used time-resolved fluoroimmunoassay (TR-FIA) to analyze estradiol and progesterone concentrations in fecal samples. We measured fecal samples taken during estrus period, diestrus period, pregnancy and non-pregnancy period. During estrus (February), the mean minimum estradiol concentration was 4.02 ± 1.9 ng/g, and the mean maximum was 86.01 ± 35.2 ng/g (dry fecal weight). During diestrus (November), the mean minimum estradiol concentration was 4.42 ± 1.32 ng/g and mean maximum was 15.62 ± 6.48 ng/g (dry fecal weight). Midgestation (April), the mean minimum progesterone concentration was 427 ± 24.49 ng/g and the mean maximum was 1490 ± 265.27 ng/g. During non-pregnancy (November), the mean minimum progesterone concentration was 71.25 ± 29.61 ng/g and the mean maximum was 291.75 ± 90.30 ng/g. These results suggest that steroid hormone analysis of feces using TR-FIA is a valid method for noninvasively determining ovarian activity associated with estrus and pregnancy in leopard cats. This study will contribute to building breeding management and reproductive plans for endangered species.

Key words **:** leopard cat, *Prionailurus bengalensis*, TR-FIA, estradiol, progesterone.

Introduction

The leopard cat (*Prionailurus bengalensis*) has the broadest geographic distribution of all the small Asian cats (15,18). Leopard cat living in Korea has been designated a grade II endangered wild animal by the Ministry of Environment since 1998.

Fecal steroid metabolite monitoring is a well-established tool for evaluating reproductive processes in diverse mammalian species, including felids. Fecal hormone monitoring is one of the most powerful tools available in zoo research today (1). Conventional methods for obtaining normative endocrine data in domesticated animals have relied upon analysis of serially collected blood samples. This approach is impractical for most non-tractable and stress-susceptible wildlife species, including felids (2). For the purpose of noninvasive animal hormone monitoring, radioimmunoassay (RIA), enzyme immunoassay (EIA), fluoroimmunoassay (FIA), high-performance liquid chromatography (HPLC), and gas chromatography/mass spectrometry (GC/MS) have been successfully used to measure the hormones of cheetahs (3), tigers, lions, caracals (6), leopard cats, and clouded leopards (2).

TR-FIA is used for humans commercially, and this technique offers the advantages of high sensitivity, stability of the reagents, lack of radiation, low background interference, and a wide test range (12). Time-resolved fluoroimmunoassay (TR-FIA) is a novel, alternative method to the traditional tests. It has been successfully used for measuring concentrations of porcine relaxin (11), monitoring luteal function in the sika doe (16), and determining progesterone levels in Iberian red deer (13).

This study was done to confirm the basic standard of reproductive physiology through the analysis of estradiol and progesterone from fecal sample of leopard cats during the estrus and diestrus periods, pregnancy period, and non-pregnancy period. Additionally, it was done to validate to the viability of the TR-FIA method.

Materials and Methods

Experimental animals and fecal sample collection

Fecal samples were collected from four female leopard cats (designated A, B, C and D) raised in a zoo. Each of them were raised on different isolated facilities. With four leopard cats, sampling was performed 11 times in February (estrus period), 9 times in March (pregnancy period), and 8 times in November (diestrus and non-pregnancy period) through 2006. One of 4 leopard cats (B) was taken a sample 63 times during a year period from September 2005 to August 2006. Collected samples were frozen for conservation.

Extraction of steroid metabolic materials from fecal samples

When extracting steroid metabolite material from fecal samples, the samples were freeze-dried to minimize interference

[1]Corresponding author.
E-mail : nsshin@snu.ac.kr, bjlee@chonnam.ac.kr

from moisture, following Wasser *et al.* (17). Freeze-drying was performed with a freeze dryer (FDU-2100, EYELA, Japan) for 48 hours, to evaporate fecal sample moisture under freezing condition. Once the freeze-drying step was completed, the samples were conserved in a deep freezer at −70ºC. Freeze-dried samples were refined by the elimination of hay, hair, bone, and so forth, and ground to powder on a clean bench. Fecal powder was contained with 15 ml conical tube and conserved freezing before experiment.

Steroid meabolites were extracted with ether, following Hamasaki *et al.* (8). Vortexing was done of 0.2 g of frozen fecal powder with 2 ml of phosphate-buffered saline (PBS) and 10 ml of ether, making the total volume 12 ml. The samples were then shaken for 10 minutes with a Belly Dancer mixing platform (Stovall Life Science, USA) at room temperature. Centrifuging was done at 4ºC at 1500 rpm for 10 minutes, and samples were kept overnight in a −70ºC deep freezer. Ether was poured onto a layer of the sample in another conical tube and evaporated with nitrogen gas in a 33ºC evaporator (EYELA, MG-2200, Japan). After evaporation, 1 ml of Tris buffer (50 mM Tris-HCl, pH 7.8) was added to the remnant in the conical tube, and the sample was vortexed and then conserved in a −20ºC in deep freezer.

Steroid hormone analysis with TR-FIA

There was no species-specific TR-FIA kit for the steroid hormone of leopard cat, so human TR-FIA kits (DEFIA estradiol and DEFIA progesterone kits, PerkinElmer, Finland) were used in this study.

Frozen samples were kept at room temperature for 30 minutes. After that, they were again kept at room temperature for 30 minutes after adding 1 ml of saline both to the estradiol standard seven vials (A-G) in the DEFIA estradiol kit and to the progesterone standard six vials (A-F) in the DEFIA progesterone kit. Antiserum (estradiol-antiserum stock solution) in the kit was diluted 10 times with estradiol assay buffer and progesterone assay buffer. Washing buffer was made with 40 ml of the kit's washing concentrate and diluted 25 times, using a washer system (DELFIA plate wash, Wallac, Finland), and replaced every 2 weeks. Estradiol plates were washed once before use and progesterone plates four times before use.

Standard buffer and Tris buffer were poured, 25 µl into each of two wells, and samples were poured into two wells, also. Diluted antiserum solution was poured, 100 µl into each well, and shaking incubation was performed using the Belly Dancer for 30 minutes at maximum speed and room temperature.

Tracer (estradiol-Eu stock solution) was diluted 50 times with the estradiol and progesterone assay buffers. Diluted tracer solution was poured, 100 µl into each well, and shaking incubation was performed for 2 hours in the Belly Dancer at maximum speed and room temperature. Washing was performed six times for the analysis of estradiol and four times for the analysis of progesterone.

Enhancement solution was poured, 200 µl into each well, and shaking incubation was performed in the Belly Dancer for 5 minutes at room temperature. The sample was measured and repeted twice with a time-resolved fluorometer (VICTOR^{2}D, PerkinElmer, Finland), After that, analysis conducted with a Wallac MutiCac program (Wallac, Finland). Result from analysis, valid detection range of estradiol was 0-16.4 nmol and progesterone was 0-136 nmol. During the analysis, the measurement results that had variation over 15% of the detection range were excepted from statistical analysis.

Results

The results of fecal steroid metabolic material analysis of four leopard cats with a TR-FIA kit, in the estrus period (February) for A, B, C, and D, yielded a mean minimum estradiol concentration of 4.02 ± 1.9 ng/g and a mean maximum of 86.01 ± 35.2 ng/g (dry fecal weight). In the diestrus period (November) for A, B, C, and D, the mean minimum estradiol concentration was 4.42 ± 1.32 ng/g, and the mean maximum was 15.62 ± 6.48 ng/g (dry fecal weight) (Table 1).

In the middle of pregnancy (March) for A, B, C, and D, the mean of the minimum range of progesterone concentrations was 427 ± 24.49 ng/g (dry fecal weight), and the mean of the maximum range was 1490 ± 265.27. During the non-pregnant period (November) for A, B, C, and D, the mean of the minimum range of progesterone concentrations was 71.25 ± 29.61 ng/g, and the mean of the maximum range was 291.75 ± 90.30 ng/g (Table 2).

Leopard cat B, whose fecal samples were collected for 1 year, was in estrus in February, and gave birth on April: there was no estrus cycle in the fall. Its progesterone concentrations during pregnancy were much higher than those during non-pregnancy (Fig 1).

Discussion

Fecal progesterone levels increased markedly at 20ºC (but not at 4ºC). Therefore it appears that the increase was caused by the action of intestinal microorganisms, which converted conjugated steroids to unconjugated forms (19). In this study, we did not estimate the effects of microorganism on progester-

Table 1. Fecal estradiol concentrations (ng/g dry fecal weight) during estrus and diestrus periods

Individuals	Estrus Period (February)		Diestrus Period (November)	
	Basal	Peak	Basal	Peak
A	6.86	83.63	3.81	8.5
B	3.02	132.66	2.86	11.5
C	3.49	80.63	5.63	23.4
D	2.70	47.12	5.39	19.1
Mean	4.02 ± 1.9	86.01 ± 35.2	4.42 ± 1.32	15.62 ± 6.84

Basal = lowest value, Peak = highest value
Numbers of individual fecal samples in February were 11 times, and samples in November were 8 times.

Table 2. Fecal progesterone concentration (ng/g dry fecal weight) during pregnancy and nonpregnancy periods

Individuals	Progesterone Concentration during Pregnancy (March)		Progesterone Concentration during Non-pregnancy (November)	
	Basal	Peak	Basal	Peak
A	433	1786	114	379
B	439	1152	49	187
C	391	1576	68	354
D	445	1447	54	247
Mean	427 ± 24.49	1490.25 ± 265.27	71.25 ± 29.61	291.75 ± 90.30

Basal = lowest value, Peak = highest value.
Numbers of individual fecal samples in March were 9 times, and samples in November were 8 times.

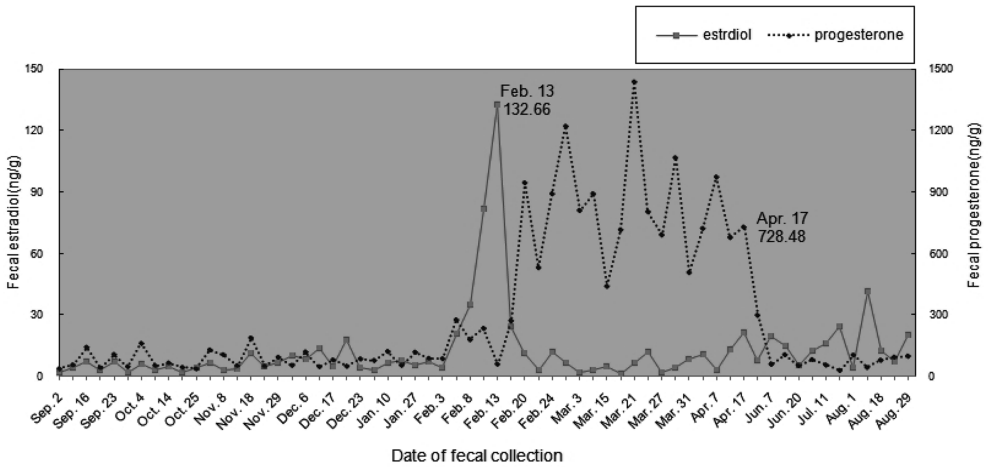

Fig 1. Longitudinal profiles of fecal estradiol (unbroken line) and progesterone (dotted line) of single leopard cat B, from September 2005 to August 2006. The estradiol peak was on February 13, 2005, at 132.66 ng/g; the progesterone level sharply declined on April 19, after parturition.

one concentration, but mostly fresh samples were collected, which were kept immediately in a deep freezer to minimize the effects of microorganisms.

Data were variable and apparently influenced by the amount and distribution of hair throughout the samples (2). We eliminated foreign bodies like soil or hair when samplings were performed. There were abnormal results in that estradiol or progesterone ranges were unusually high; it was suspected that the effects of minute particles of hay, hair, soil, and so forth could not be eliminated before analysis.

Fecal E2 concentrations in the leopard cats increased 30-fold over the baseline within 3 days of gonadotropin injection. In clouded leopards, approximately 5-fold increases in estradiol were observed during estrus, compared to the baseline. Again, after estrus, estradiol excretion returned to the baseline (2). In this study, the estradiol concentrations of the five leopard cats increased 21-fold, from 4.02 ± 1.9 ng/g to 86.01 ± 35.2 ng/g (peak day) during the estrus season (February).

These observations indicate that increased estradiol concentrations are associated with estrus. Therefore, it can be surmised that more than 86.01 ± 35.2 ng/g (dry fecal weight) estradiol concentrations in some leopard cats indicate that these animals are in the estrus and ovulation stages.

Noninvasive endocrine monitoring has been applied to free-ranging animals as well as captive animals (7). Pregnancy diagnosis has been achieved noninvasively in free-ranging elk, *Cervus elaphus nelsoni* (5) and African elephants (4). Fecal progesterone metabolite analysis has been successfully used for monitoring the corpus luteum function and the pregnancy, abortion, and puberty in an expanded number of species, including primates (14). Fecal P4 metabolite concentrations of the leopard cats studied here were at their lowest level before artificial insemination (AI), but they increased within 5 days after AI, and were highest (a 100-fold increase) between days 5 and 35 of pregnancy. Although this elevation lasted throughout gestation, P4 concentrations gradually declined after midgestation, reaching the baseline within 2 weeks postpartum (2). In this study, there was a difference in the means of maximum progesterone concentrations between the March midgestation period (1490.25 ± 265.27 ng/g dry fecal weight) and the November non-gestation period (291.75 ± 90.30 ng/g dry fecal weight); the difference was more than 5-fold (Table 2). Given this result, the analysis of progesterone concentrations in feces could be a useful pregnancy test.

Leopard cat B was sampled for 1 year, began estrus in February, had a gestation period of 65 days, and experienced parturition in mid-April. There was no estrus in the fall. These things indicate that the leopard cat is a seasonal breeder, which coincides with previous reports that these animals come into heat in March. Gestation, as in other small cats, requires about 60 days. Kittens appear in the second half of May (9).

Use of human assays or human reference laboratories for assaying animal specimens is unwise, unless the assays have been adequately validated (12). Care must be taken to validate noninvasive endocrine-monitoring techniques for each species of interest (7). The results of this study demonstrate that the commercial TR-FIA kits used for humans can be usefully applied in the case of leopard cat. Estradiol and progesterone concentration tests using TR-FIA may be judged a useful method for the basic study of reproductive physiology and pregnancy testing of wild animals that are not captive or are otherwise difficult to approach.

References

1. Brown JL, Graham LH, Wielebnowski N, Swanson WF, Wildt DE, Howard JG. Understanding the basic reproductive biology of wild felids by monitoring of faecal steroids. J Reprod Fertil Suppl 2001; 57: 71-82.
2. Brown JL, Wasser SK, Wildt DE, Graham LH. Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured noninvasively in feces. Biol Reprod 1994; 51: 776-786.
3. Brown JL, Wildt DE, Wielebnowski N, Goodrowe KL, Graham LH, Wells S, Howard JG. Reproductive activity in captive female cheetahs (Acinonyx jubatus) assessed by faecal steroids. J Reprod Fertil 1996; 106: 337-346.
4. Foley CAH, Papageorg S, Wasser SK. Noninvasive stress and reproductive measures of social and ecological pressures in free-ranging African Conservation Biology 2001; 15: 1134-1142.
5. Garrott RA, Monfort SL, White PJ, Mashburn KL, Cook JG. One-sample pregnancy diagnosis in elk using fecal steroid metabolites. J Wildl Dis 1998; 34: 126-131.
6. Graham LH, Raeside JI, Goodrowe KL, Liptrap RM. Measurements of faecal oestradiol and progesterone in non-pregnant and pregnant domestic and exotic cats. J Reprod Fertil Suppl 1993; 47: 119-120.
7. Graham LH. Noninvasive monitoring of reproduction in zoo and wildlife species. ARBS Ann Ver Biomed Sci 2004; 6: 91-98.
8. Hamasaki S, Yamauchi K, Ohki T, Murakami M, Takahara Y, Takeuchi Y, Mori Y. Comparison of various reproductive status in Sika deer (Cervus nippon) using fecal steroid analysis. J Vet Med Sci 2001; 63: 195-198.
9. Heptner VG, Sludskii AA. Carnivora (Hyaenas and cats). In: Mammals of the Soviet Union, New Delhi: Amerind Publishing. 1992: 346-354.
10. Nowak RM. Walker's Mammals of the World, 6th ed. Maryland: Johns Hopkins University Press. 1999: 812-813.
11. Ogine T, Kohsaka T, Taya K. Time-resolved fluoroimmunoassay (TR-FIA) of porcine relaxin. Exp Clin Endocrinol 1999; 107: 276-280.
12. Parra MD, Bernal LJ, Cerón JJ. Cortisol and free thyroxine determination by time-resolved fluorometry in canine serum. Can J Vet Res 2004; 68: 98-104.
13. Parra MD, García AJ, Bernal LJ, Landete-Castillejos T, Cerón JJ. Progesterone determination in Iberian red deer by time-resolved fluorometry: an alternative method to RIA. J Exp Zoolog A Comp Exp Biol 2004; 301: 472-476.
14. Schwarzenberger F, Mostl E, Palme R, Bamberg E. Faecal steroid analysis for noninvasive monitoring of reproductive status in farm, wild, and zoo animals. Anim Reprod Sci 1996; 42: 515-526.
15. Sunquist M, Sunquist F. Wild Cats of the World. Chicago: The University of Chicago Press. 2002; 226-232.
16. Takahashi T, Hamanaka S, Imai K, Hashizume K. Fecal progesterone analysis by time-resolved fluoroimmunoassay (TR-FIA) for monitoring of luteal function in the sika doe (Cervus nippon centralis). J Vet Med Sci 2002; 64: 565-569.
17. Wasser SK, Monfort SL, Southers J, Wildt DE. Excretion rates and metabolites of oestradiol and progesterone in baboon (Papio cynocepbalus cynocepbals) faeces. J Reprod Fertil 1994; 101: 213-220.
18. Wilson DE, Reeder DM. Mammal Species of the World: A Taxonomic and Geographic Reference, 3rd ed., Vol. 1. Maryland: Johns Hopkins University Press. 2005; 542-543.
19. Yamauchi K, Hamasaki S, Takeuchi Y, Mori Y. Application of enzyme immunoassay to fecal steroid analysis in sika deer (Cervus nippon). J Reprod Dev 1999; 6: 429-434.

삵에서 TR-FIA를 이용한 분변내 Estradiol과 Progesterone의 검사

김영섭* · 김지용** · 정소영* · 이봉주***[1] · 신남식**[1]

* 서울대공원, **서울대학교 수의과대학, ***전남대학교 수의과대학

요 약 : 본 연구는 총 4두의 삵에서 발정기와 비발정기, 임신기와 비임신기 동안의 분변에서 steroid metabolic materials를 추출한 후, TR-FIA kit를 이용하여 estradiol과 progesterone의 농도를 측정하였다. 발정기간 (2월) 중 estradiol 농도의 최저는 평균 4.02 ± 1.9 ng/g 이었고, 최고는 평균 86.01 ± 35.2 ng/g (dry fecal weight) 이었다. 비발정기 (11월)의 최저는 평균 4.42 ± 1.32 ng/g 이었고, 최고는 평균 15.62 ± 6.84 ng/g 이었다. 임신기 (3월) 중 progesterone농도의 최저는 평균 427 ± 24.49 ng/g 었고, 최고는 평균 1490 ± 265.27 ng/g 이었다. 비임신기 (11월)의 progesterone의 최저는 평균 71.25 ± 29.61 ng/g 이었고, 최고는 평균 291.75 ± 90.30 ng/g 이었다. 위의 결과에 따라 삵에서 TR-FIA에 의한 분변내 steroid hormone 의 측정은 발정과 임신에 관련된 난소활동을 비 침습적으로 평가하기 위한 적절한 방법으로 판단되었다. 본 연구는 삵과 같이 접근이 어렵고 멸종위기에 처한 야생동물의 사육관리의 효율화와 번식계획의 수립에 도움이 될 것이다.

주요어 : 삵, *Prionailurus bengalensis,* TR-FIA, estradiol, progesterone