Fecal Progesterone Analysis by Time-Resolved Fluoroimmunoassay (TR-FIA) for Monitoring of Luteal Function in the Sika Doe (Cervus nippon centralis)

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ABSTRACT. Fecal progesterone content was measured by time-resolved fluoroimmunoassay (TR-FIA) in the sika doe (Cervus nippon). The total recovery rate of fecal progesterone by twice extraction with diethylether was about 60%. The displacement curve of TR-FIA with serial doses of fecal extract (0.156–5.0 mg feces) was closely parallel to that of the reference standard. Fecal progesterone content was correlated with that of plasma (r=0.829, n=16), but the values were 100-fold higher in feces than in plasma. Fecal progesterone content periodically changed during the breeding season suggesting the estrous cycle in the doe. The fecal progesterone content was higher between the estruses, and decreased after estrus. The time between the onset of estrous signs and the lowest fecal progesterone content was 1–2 days suggesting the time required for hepatic metabolism and intestinal passage. Fecal progesterone content was also decreased around the time of vaginal discharge. The discharge took place in few days, suggesting a short luteal phase. Not all decreases in fecal progesterone values were preceded by estrous behavior or vaginal discharge. Fecal progesterone content was further increased in pregnancy rather than in the preceding estrus cycle and the levels were maintained up to term. These results suggest that fecal progesterone measurement is a useful tool for non-invasive analysis of luteal function in the sika doe. The TR-FIA kit, designed for the human hospital market, was shown to be successfully utilized for fecal assay in the sika doe with minor modifications.

KEY WORDS: breeding season, fecal progesterone, pregnancy, sika deer, TR-FIA.
labeled tracer. The TR-FIA is based on time-resolved fluorometry instead of radioactivity counting [10]. This assay has been applied for plasma progesterone analysis in the sika deer [20].

In the present study our objective was focused on the establishment of fecal progesterone analysis for determination of luteal functions in the sika deer. This paper provides simple procedure for fecal progesterone analysis with TR-FIA and describes the profiles of fecal progesterone content of sika does in the breeding season and subsequent gestation.

MATERIALS AND METHODS

Animals and blood sampling: Sika does, of various ages and parities, housed in the Miyagi Prefecture Agricultural College (Sendai, Japan, 38N, 141E), were used for this study. The animals were fed hay, commercial concentrate feed and tap water and kept without restraint except when sampling blood. The reproductive behavior was monitored by observing the animals at least once a day throughout the breeding season.

Blood samples were collected via jugular venipuncture after intramuscular blow injection of an anesthetic agent (Xylazine; 1 mg/kg body weight). The collected blood was placed on ice immediately after bleeding and the plasma was separated on a centrifuge within 1 hr of collection. Blood sampling was done monthly in non-breeding seasons (April to October 1999 and February to May 2000) and twice a month in the breeding season (November 1999 to January 2000). Fecal samples (5–10 g) were collected immediately after defecation every second day. Harvested plasma and feces were stored at –20°C until assayed.

Progesterone extraction from feces: The procedure of progesterone extraction from feces is shown in Fig. 1. The stored samples were thawed at room temperature. The 0.1 g and 0.5 g of fresh feces were used for progesterone extraction and dry matter estimation, respectively. The 0.1 g of fresh feces was homogeneously suspended in 2 ml of phosphate-buffered saline (PBS). The 1/10 fecal suspension (200 µl) was resuspended in 2 ml of PBS and extracted by vortex mixing for 3 min with 5 ml of diethyl ether. After separation of the 2 phases, the aqueous layer was snap frozen by soaking in a dry ice-cold ethanol bath. The organic layer was decanted into glass test tubes and evaporated under nitrogen gas. The dried samples were dissolved in 200 µl of assay buffer. Fifty µl of the reconstituted sample was used for TR-FIA. To assess the extraction efficiency, eleven fecal samples were serially extracted 5 times to estimate cumulative rates of extraction.

For the assessment of dry matter content in individual samples, 0.5 g of fresh feces was dried on aluminum foil in an oven at 65°C for 24 hr, then further dried at 135°C for 2 hr prior to subtractive weighing. The fecal progesterone values were shown as content per gram of dried feces. Fecal progesterone content was calculated as follow: Content = assay value/extraction rate (0.6)/dry matter ratio.

RESULTS

Validation of progesterone assay: Figure 2 shows the mean cumulative recoveries (mean +/- SEM) of progesterone from 11 samples after repeated extraction. The recovery rate was calculated from the progesterone content in individual samples, as the sum total of quintuple extraction was 100%. Maximal recovery was extrapolated as 110% after limitless extraction. The result in Fig. 2 shows that 70.1% of progesterone was recovered by twice extraction. The total recovery of progesterone from feces after twice extraction was calculated as 70.1/110 = 0.637. The rate was rounded to 0.6 and used for calculation in the next progesterone assay. The parallels between the displacement curve of the reference standard and serial doses of fecal extract are shown in Fig. 3. In the doses tested, displacement curve of the fecal extract closely paralleled that of the reference standard and serial doses of fecal extract was confirmed. The plasma progesterone concentrations were measured directly by TR-FIA without extraction as previously described [20]. The progesterone assays of both fecal and plasma samples were done in duplicate. The minimum detectable level of TR-FIA was 7.75 pg/well. The intra- and inter-assay coefficients of variance were 7.3% and 10.1%, respectively.
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The regression formula was as follow; \( Y=107.0X+73.4 \) (n=16, Y=fecal content; ng/g dried feces, X=plasma concentration; ng/ml, correlation coefficient; \( r=0.829 \)). Specimens used for this experiment were collected in the luteal phase (n=3), gestation (n=6) and anestrus (n=7).

Monitoring of fecal progesterone profiles in the doe: The fecal progesterone profiles of 2 sika does in the breeding season are shown in Figs. 5-A and 5-B. Fecal progesterone content fluctuated periodically during the breeding season. The content was higher in between the estruses, and lower around estrus. The estrous behavior and vaginal discharge were observed on 1 to 2 days prior to the time of the lowest fecal progesterone content. The result shown in Fig. 5-B indicates that fecal progesterone content was decreased at the time of vaginal discharge suggesting multiple short cycles. The length of the estrous cycle in the sika doe, which was estimated by the interval between the lowest fecal progesterone content is 17 and 19 days (Fig. 5-A). But from the data in Fig. 5-B, the length of the fecal progesterone cycle ranged 9–13 days.

Plasma and fecal progesterone profiles in pregnant sika doe are shown in Fig. 6. This animal showed estrous signs on both November 1 and 19 in 1999 and delivered newborn on July 7 in 2000. Serial fecal sampling lasted until December 21 in 1999, then monthly profile of fecal progesterone was monitored until 2 days before delivery. Fecal progesterone content reached its minimum on November 20, thereafter increased. Fecal progesterone content further increased after January 2000, and thereafter the values were maintained up to the term of gestation. Plasma progesterone concentrations were lower than 2 ng/ml in November but higher than 3 ng/ml from December 1999 to May 2000. The progesterone profile in plasma coincided with that of feces. Plasma samples were not collected in June or July to avoid unexpected trouble with the anesthetic agent.

**DISCUSSION**

The results of the present study showed the correspondence between progesterone values for plasma and feces, suggesting that fecal progesterone measurement could be used for the sika doe in non-invasive analysis of luteal functions. The rate of extraction of progesterone from feces was similar to that in the previous report in the shiba goat [8], which showed that about 70% of progesterone was extracted.
from ground feces following desiccation. By contrast, in the present study, about 60% of progesterone was extracted from raw feces. Since the grinding of every fecal sample involves considerable inconvenience, especially when there were many samples, we used raw feces for progesterone measurement without desiccation and grinding. The extraction might be affected by the sample status (dried or raw) and the procedure (ether volume and mixing time). Since it has certain advantages for routine sample preparation, progesterone extraction from raw feces could be utilized for fecal steroid analysis.

Fecal progesterone content decreased and reached its lowest on 1–2 days after estrus. The time lag in progesterone profiles of plasma and fecal samples was reported in other species with intact and progesterone-implanted animals [8]. Plasma progesterone is secreted into intestine as bile after hepatic metabolism and some progesterone is returned to the liver by enterohepatic circulation [17]. The time for both hepatic metabolism and intestinal passage of bile to the rectum, will cause a lag in progesterone profiles of plasma and fecal samples.

The progesterone content in goat feces was reported to be gradually increased after defecation at room temperature [8]. This phenomenon is thought to be a conversion of conjugated progesterone into the unconjugated form by intestinal microflora, although details of the mechanisms are still obscure [22]. Actually it is impossible to collect feces immediately after defecation in the field, but it is reported that fecal progesterone content in the goat was scarcely increased at room temperature within several hours after defecation [8]. And the increase in fecal steroid content, when feces are left at ambient temperature, is inhibited by a cold environmental as well as by the addition of ethanol, antibiotics and silica gel [22]. Taking account of this finding, fecal progesterone analysis could provide reliable data, especially in winter, in determining luteal function in the wild deer.

Fecal progesterone content in the sika doe changed periodically during the breeding season. The results of the present study suggest that fecal progesterone analysis could be utilized to determine seasonal breeding in the sika deer, but the length of the fecal progesterone cycle of individual deer differed. In fallow deer there has been reported silent ovulation associated with short-lived corpora lutea at the beginning of the season [2]. Estrous behavior and vaginal discharge were followed by a decrease in fecal progesterone content, but not every valley in the fecal progesterone pro-

Fig. 5. Fecal progesterone profiles during the breeding season. (A) doe with regular luteal cycles 17 and 19 days long and (B) doe with multiple short-lived corpora lutea 9–13 days long as indicated by transitory increases in fecal P. Arrows show the estrous behavior. Arrowheads show the mucus discharge from the vulva.

Fig. 6. Fecal and plasma progesterone profiles during the breeding season and subsequent pregnancy. Fecal (■) progesterone profile was monitored by sampling every second day in the breeding season and monthly in pregnancy. Plasma (●) progesterone profile was monitored by biweekly sampling in the breeding season and monthly in pregnancy. JFMAMJJ shows calendar months from January to July in 2000. Plasma samples were not collected on June and July to avoid unexpected trouble with the anesthetic agent. Arrows show the estrous behavior.
file was preceded by estrous behavior. This phenomenon will be associated with either silent ovulation or failure to detect estrus. In the sika doe, since estrous detection is relatively difficult rather than in other domestic species, the reason for a fecal progesterone cycle without estrous signs remains to be elucidated.

As pregnant does had high fecal progesterone content which was maintained until just before parturition. Fecal progesterone assay may be a reliable way to diagnose pregnancy in the sika doe. For precise diagnosis, the combined analyses of fecal progesterone and other placental steroids could be more effective.

In conclusion, our findings from the limited number of animals, suggest that fecal progesterone content in the sika doe is correlated with both the plasma progesterone concentration and reproductive behavior. Fecal progesterone analysis is suggested to be a suitable tool for monitoring of luteal functions in the sika deer, but further study is needed to confirm and clarify this.

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REFERENCES