—Research Note—

Changes in Fecal Progestagen Profile After Excretion in Miniature Pigs

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Abstract. The aim of this study was to evaluate whether the fecal progestagen (progesterone and its metabolites) levels of miniature pigs would change after excretion at room temperature. Our initial investigation focused on the correlations between the fecal progestagen concentrations with and without ether extraction and between the plasma progesterone and fecal progestagen concentrations in order to develop an enzyme-linked immunosorbent assay (ELISA) for fecal progestagen without ether extraction. There were significant correlations between fecal progestagen concentrations with and without ether extraction ($r=0.880$) and between fecal progestagen concentrations without ether extraction and plasma progesterone ($r=0.763$). The fecal progestagen concentration obtained by ELISA without ether extraction was almost identical to that obtained with ether extraction. These results validate the ELISA method without ether extraction, which was therefore used for the latter experiment. Fecal samples collected from the pigs were preserved for 0–24 h at room temperature, and then their fecal progestagen concentrations were measured. The fecal samples preserved for 0 to 24 h were analyzed by high performance liquid chromatography (HPLC) and ELISA. The concentrations of all samples significantly increased with time after preservation. The progestagen concentration of fresh feces (0 h) with high progestagen concentration (>1000 ng/g) increased significantly after 3 h. The concentration increased significantly after 12 h for fresh feces containing about 500 ng/g progestagen. HPLC analysis is showed that the fecal progesterone concentration, but not its other metabolites, doubled 24 h after excretion compared with the concentration at 0 h. These results suggest that dynamic changes in the profile of progesterone metabolites occur in feces after excretion.

Key words: Enzyme-linked immunosorbent assay (ELISA), Feces, Pig, Progestagen, Progesterone
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To monitor ovarian function in animals, the progesterone concentration is usually measured using plasma and serum [1–3]; however, blood sampling entails retraining the animal and insertion of a needle into it, both of which induce stress. These stressors possibly interfere with the precise timing of reproductive hormone release within the follicular phase and possibly reduce the productivity of animals [4]. These problems can be

overcome by using feces to measure the steroid hormone level. Fecal progestagen (progesterone and its metabolites) analyses have been used to diagnose pregnancy in sows [5], cows [6] and primates [7], and to monitor ovarian function in sows [8, 9], beef cattle [10], baboons [11] and black rhinoceroses [12].

Fecal sampling is a non-invasive method for determining reproductive status and is easier than blood sampling. Assays for fecal progestagen usually require an extraction procedure with organic sorbent, which complicates the progestagen assay.

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If the extraction procedure is omitted, the hormone assay is rapid and simple.

Fecal samples are usually collected from the rectum and used for hormone assays of progesterone [13, 14], estrone derivatives [15] and corticoid [16]; however it is difficult, laborious and dangerous to collect fecal sample from the rectum of large wild animals [12, 17], and defecated feces are therefore more convenient.

Some studies have investigated the effects of storage on fecal hormone concentration [18–20]. Galama [12] reported that there was no difference in the fecal progesterone concentrations of black rhinoceroses between 0 and 30 days of storage without preservatives. On the other hand, Wasser [7] reported that the fecal progesterone levels of feces from primates without a preservative were significantly elevated 6 h after excretions; however, little analysis of the time-dependent changes in the steroid hormone concentrations of feces after excretion has been conducted.

The miniature pig is preferable for laboratory studies because of its small body size compared with commercial species such as Landrace and Yorkshire pigs. However, it is not clear whether the changes in the fecal progestagen concentrations of the miniature pig are similar to those of commercial species.

The present study examined an easy assay procedure without ether extraction and investigated whether the fecal progestagen levels of miniature changed after defecation.

Materials and Methods

Animals and sample collection

Göttingen miniature pigs (four pregnant and four non-pregnant pigs), 0 to 5 years old and weighing approximately 50 kg, were used in this experiment. The pigs were checked each day for estrous behavior by a technician (observation of vulvar signs and standing heat). The animals were fed twice daily with a commercial feed and were allowed free access to water in accordance with the Animal Care/Ethics Committee of Hiroshima University. The feed contained 15% DCP and 74% TDN. Fecal samples were collected once a week from the rectum with a nylon-covered finger, placed in a nylon bag and stored at -20°C until the assay. Blood samples from three of the non-preg-

nant miniature pigs were collected from the jugular vein into heparinized vacuum tubes. The blood and fecal samples were collected simultaneously to correlate the analysis of the progesterone and progestagen levels. The blood samples were centrifuged ($1,700 \times g$, 15 min, 5°C) and the blood plasma samples were stored at -20°C until the assay.

Extraction of feces

The first extraction of feces with methanol was conducted as described by Isobe and Nakao [10]. A fecal sample (0.3 g) and 18 ml of methanol were added to polypropylene tubes, shaken for 15 min and then centrifuged at $1,700 \times g$ for 5 min. The supernatant fecal solution was stored at 5°C until the assay.

The fecal solution (20 μl) or plasma (20 μl) supplemented with double distilled water (100 μl) was extracted with petroleum ether (3 ml). The ether phase was decanted into another tube and evaporated. Progestagen and progesterone were suspended again by the addition of borate buffer [0.05 M boric acid, 0.2% BSA (fraction V; Sigma-Aldrich, St. Louis, MO, USA) and 0.1 mg/ml thimerosal].

Progestagen assay

The progestagen and progesterone concentrations in feces and plasma, respectively, were measured by enzyme-linked immunosorbent assay (ELISA) as described by Isobe and Nakao [21]. The standard solution was prepared with borate buffer at concentrations of 0, 0.037, 0.1, 0.3, 1 and 3 ng/ml of progesterone. Ether-extracted and unextracted fecal solutions and ether-extracted blood samples were added to the duplicate wells of a microtiter plate previously coated with goat anti-rabbit IgG antibody (ICN Biomedicals, Aurora, OH, USA). The antibody against progesterone-3-carboxymethyloxime conjugated to BSA was produced in a rabbit [1] and its cross-reactivity with progesterone, 5 α -pregnanedione, 20 β -hydroxyprogesterone, deoxycorticosterone, pregnenolone, 5 β -pregnane-3 α -ol-20-one and 17 α -hydroxyprogesterone was 100, 5.8, 0.7, 0.62, 0.2, 0.1 and 0.05%, respectively. This antibody and horseradish peroxidase-labeled progesterone [10] were applied to the wells followed by incubation at room temperature for 2 h. After washing with PBS three times, o-phenylenediamine solution (4 mg/ml o-phenylenediamine, 0.2 M citric acid and 0.02%

H₂O₂) was added to the wells. After incubation at room temperature for 30 min in the dark, the reaction was stopped by the addition of 6 N H₂SO₄. Optical density was measured using a microplate reader (Model 550; Bio-Rad Laboratories, Hercules, CA, USA) at 492 nm.

HPLC

The feces contained various progesterone metabolites and authentic progesterone; therefore, Isobe *et al.* [10] extracted bovine fecal solution with petroleum ether to omit progesterone metabolites. To check this, we separated the fecal samples before or after extraction with petroleum ether and fresh or preserved samples using a reverse-phase Capcell Pak column (2.0 × 100 mm, Shiseido, Tokyo, Japan) as described by Shimada and Terada [22]. The solvent delivery system contained 50% (v/v) methanol solution. Thirty fractions (2 min per fraction.) were assayed for progestagen using this method. The flow rate was adjusted to 200 μ l / min. To confirm the fraction of progesterone, authentic progesterone was also assayed.

Changes in the progesterone metabolite profile after fecal excretion

The fecal samples collected from the 8 pigs were weighed and preserved at room temperature. After preservation for 0, 1, 3, 6, 12 and 24 h the feces were weighed again, extracted with methanol and assayed for their progestagen content. The moisture content of the feces gradually decreased as the preservation time increased. Therefore, the corrected fecal progestagen value was calculated to compare the fresh and preserved feces. The calculation formula was as follows: Corrected value (ng/g) = [fecal progestagen concentration (ng/g) × weight of feces preserved for given time (g)] / [weight of fresh fecal sample (g)].

Statistical analysis

Correlation analysis was carried out using Spearman's correlation analysis between fecal progestagen with and without ether extraction, between fecal progestagen without ether extraction and plasma progesterone, and between fresh and preserved fecal progestagen followed by analysis of the significance of the correlation coefficient by ranks. The correlation was calculated for each animal as well as for all animals in order to avoid animal-specific variation in correlation. The cor-

rected fecal progestagen concentrations for various preservation periods were compared by one-way ANOVA followed by Duncan's multiple range test (StatView; Abacus Concepts, Berkeley, CA, USA).

Results

The assay sensitivity of the progesterone ELISA was calculated as the concentration of progesterone that can detect two standard deviations below the mean optical density at 0 ng/ml progesterone. The value was estimated to be 32.5 pg/ml. The standard curve was identical to the curve of the fecal serial dilution. The recovery rates of ELISA with or without ether extraction after addition of 1–5 ng/ml progesterone to feces and extraction with methanol were 88.8–101.6% and 87.4–96.1%, respectively. The intra- and interassay CVs for the ether-unextracted fecal samples were 6.3–6.7 (n=6) and 13.6–14.7 (n=6), and those for the ether-extracted fecal samples were 8.6–8.7 (n=6) and 12.2–12.7 (n=6), respectively.

The fecal progestagen levels of the two assay methods (with or without ether extraction) were highly correlated ($r=0.880$, $P<0.01$; Fig. 1a), although the progestagen levels without ether extraction were a little higher than the progestagen levels after ether extraction. A significantly high correlation was also obtained between the fecal progestagen level without ether extraction and plasma progesterone level ($r=0.763$, $P<0.01$, Fig. 1b).

After methanol extraction, fecal solutions with and without ether extraction were separated by high performance liquid chromatography (HPLC) and then analyzed by ELISA (Fig. 2). The fecal solutions showed the presence of four immunoreactive peaks, although three disappeared with ether extraction. The peak of fraction 17 was present in both samples and was recognized as authentic progesterone; however, the other peaks were not identified.

The changes in the corrected progestagen concentrations of feces after preservation for up to 24 h are shown in Fig. 3. The concentrations of all samples significantly increased with time after preservation. The progestagen concentrations of fresh feces with a high progestagen concentration (> 1000 ng/g) increased significantly after 3 h. The concentration of fresh feces with an initial concen-

Fig. 1. Correlations between fecal progesterone concentrations with and without ether extraction and between plasma progesterone and fecal progesterone concentrations without ether extraction.

tration of about 500 ng/g progesterone increased after 12 h. The progesterone concentration was above 500 ng/g during preservation even if the concentration of feces was < 500 ng/g prior to preservation; however, the concentration of fresh feces with an initial concentration of < 250 ng/g progesterone remained < 500 ng/g after 24 h.

The correlation between the progesterone concen-

Fig. 2. HPLC analysis of fecal progesterone before (open squares) and after (closed squares) ether extraction.

Fig. 3. Changes in the corrected progesterone concentrations during preservation for 24 h at room temperature ($n=20$). The dots indicate mean $\pm$ SD progesterone concentrations. Means with different letters (a–k) are significantly ($P < 0.05$) different in each group (a–e, f–g, h–i, j–k). Closed squares: <250 ng/g of fecal progesterone. Closed circles: >250 ng/g but <500 ng/g of fecal progesterone. Open squares: >500 ng/g but <1000 ng/g of fecal progesterone. Open circles: >1000 ng/g of fecal progesterone. A fecal progesterone concentration above 500 ng/g (dotted line) indicates the presence of a corpus luteum.

trations of feces preserved for 0 and 24 h was significant ($r=0.939$, $P < 0.01$, $n=39$, data not shown). The feces preserved for 24 h had approximately 2 times higher progesterone concentration than fresh feces.

Four immunoreactive peaks were observed during HPLC analysis of fresh feces (Fig. 4). Two of those peaks (fractions 5 and 17) were also seen in

Fig. 4. HPLC analysis of fecal progesterone at 0 (open squares) and 24 h (closed squares) after excretion.

the fecal samples 24 h after preservation, and the progesterone level of fraction 17 (progesterone) was increased approximately two-fold. Four peaks (fractions 5, 8, 10 and 14) were unknown substances. The peaks of fractions 8 and 14 in fresh feces disappeared during preservation for 24 h.

Discussion

Omission of ether extraction from ELISA is valuable for saving time and labor; therefore, we examined a direct method (without ether extraction) of measuring fecal progesterone in pigs. This method had high sensitivity and a satisfactory recovery rate. Serial dilutions of feces showed changes parallel to the standard curve of progesterone. The changes in the fecal progesterone concentration were almost identical and parallel to the plasma progesterone concentration; the correlation was significant. These results indicate that this ELISA method for fecal progesterone analysis is practical and suitable as a routine assay in pigs.

The fecal progesterone concentration obtained by ELISA without ether extraction was a little higher than that of the ether-extracted concentration. The fecal solution showed four immunoreactive peaks, although three disappeared with ether extraction; therefore, ether extraction might remove progesterone metabolites. It was reported that feces contain progesterone metabolites [23], which can be removed with an extraction procedure using petroleum ether [10]. However, there was significant correlation between the fecal progesterone concen-

trations measured by ELISA with or without ether extraction. This result validates ELISA for fecal progesterone without ether extraction.

The fecal progesterone concentrations were significantly increased in feces 24 h after excretion compared with those at 0 h. This result indicates that the amount of immunoreactive progesterone in feces begins to increase just after excretion. In fresh feces with about 500 ng/g progesterone, the concentration significantly increased after 12 h. The progesterone concentration was above 500 ng/g during preservation even if the concentration of the feces was < 500 ng/g prior to preservation. This can cause erroneous identification of a functional corpus luteum; therefore, feces taken from the rectum should be used for progesterone measurement in pigs. The same phenomenon has been observed in cattle (our unpublished data). Wasser *et al.* [7] reported that the 17 beta-estradiolB Band progestin levels of fecal samples from primates change significantly beginning 6 h after defecation; however, the fecal progesterone concentration decreases with time after excretion in cows [24]. Wasser *et al.* [7] suggested that the change in fecal progesterone is due to conversion of steroid hormones by bacteria or other microorganisms in feces. Jarvenpaa *et al.* [25] reported that the fecal steroid level is increased artificially by bacterially secreted enzymes in feces (glucuronidase).

The fecal concentration of authentic progesterone but not other metabolites, doubled by 24 h after excretion compared with 0 h. Immunoreactive peaks after the HPLC separation appeared or disappeared during preservation for 24 h. Therefore, dynamic changes in the profile of progesterone metabolites occur in feces after excretion.

In conclusion, we established a direct ELISA without ether extraction for determination of the fecal progesterone concentration in pigs. The fecal progesterone profile dramatically changed after excretion, and this should be considered carefully in field surveys for measuring fecal hormone levels.

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