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RESEARCH ARTICLE

Fecal Progestin Extraction and Analysis for Non-invasive Monitoring of Ovarian Cycle in Beef Cows

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ABSTRACT

The aims of the present study were to determine presence of immunoreactive progestins in feces, correlate fecal progestins with plasma progesterone (P_4) concentrations and subsequently assess the role of fecal progestins in monitoring estrous cycle in Kedah Kelantan (KK) beef cows. A total of 12 cycling cows were subjected to blood and matched fecal sampling twice a week for 9 weeks. The concentrations of plasma P_4 and fecal progestins extracted using a modified technique, were determined by a P_4 radioimmunoassay (RIA) kit. There was a significant positive correlation between the concentrations of fecal progestins and plasma P_4 ($r = 0.6$, $P<0.01$), as tested for the whole group except one animal. High performance liquid chromatographic separation of fecal extracts and subsequent radioimmunoassay revealed presence of four immunoreactive progestins against the P_4 antibodies. These results imply that the non-invasive measure of fecal progestins using a DSL-3900 RIA kit can be used to monitor the ovarian activity in beef cows.

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INTRODUCTION

Kedah Kelantan (KK) cows are well adapted to the tropical environment of Malaysia and have a high fertility rate. However, their beef production level needs to be improved through breeding with other better performing cattle breeds such as Brahman and Brangus, naturally or through artificial techniques. Data on reproductive hormones are useful to understand and manipulate their reproductive activity to improve their production performance through application of assisted reproductive technologies. Hormonal studies, however, traditionally involve invasive method of blood collection (Capezzuto *et al*., 2008). Although, other body fluids such as milk and urine (Schwarzenberger *et al*., 1996a) can be obtained non-invasively to determine concentrations of steroid hormones, the sampling is either limited to lactating cows (milk) or necessitates restraining the animal in a metabolic cage and putting a permanent catheter as for urine samples (Masunda *et al*., 1999).

Measurement of immunoreactive blood plasma or serum progesterone hormone (P_4) using commercial radioimmunoassay (RIA) or enzyme immunoassay (EIA) kits containing P_4 antibodies has been the most common method used to monitor reproductive activity in animals, mainly domestic species. Presence of significant cross-reactivity of P_4 antibodies of the RIA or EIA kits with several P_4 metabolites in the feces were verified based on radiometabolism studies (Palme *et al*., 1996; Isobe *et al*., 2005b; Capezzuto *et al*., 2008). Subsequently, measurements of steroid hormones including P_4 metabolites from non-invasively obtained fecal samples have been validated and established for monitoring the reproductive activity of a variety of species of animals, mainly captive and free-ranging wildlife (Schwarzenberger *et al*., 1996a; Graham, 2004; Abelson *et al*., 2009; Kummrow *et al*., 2011; Kugelmeier *et al*., 2011; Mohammed *et al*., 2011; Kinoshita *et al*., 2011; Ganswindt *et al*., 2012). Fecal sampling is non-invasive to the subject under investigation and hence it does not introduce variables that may alter results. Furthermore, it has the advantage of presenting more intensive hormone profile over a period of time with less interference and acute stress (Schwarzenberger, 2007). Owing to the relative ease of sampling and its application irrespective of the reproductive status of the animal, measuring steroid hormones from fecal samples offers great advantage and deserves to be evaluated and used in domestic animals including cattle.

Steroid hormones in feces such as progesterone residues comprise several different metabolites called progestins (Masunda *et al*., 1999). Despite several reports on the measurement of fecal progestins in cattle, due to the variation in metabolism and excretion of steroids between species (Palme *et al*., 1996; Schwarzenberger *et al*., 1996a; Palme, 2005) and even among breeds (Heistermann *et al*., 1998) and individual animals (Mohammed *et al*., 2011) of the same species as well as differences in the fecal extraction methods and immunoassays used, a direct inference in the methods from previous studies cannot be made to a particular breed like KK cows.

Therefore, the objectives of this study were to determine the presence of immunoreactive progestins in feces, the correlation between fecal progestins and plasma P_4 concentrations and to assess the use of commercial RIA method to measure fecal progestins in monitoring estrous cycle in KK cows.

MATERIALS AND METHODS

Animals: The study was carried out at the University of Putra Malaysia beef farm located at latitude of 3° North and longitude of 101° East. A total of 12 randomly selected animals (9 cycling KK cows and 3 heifers) were used. The age and parity ranged from 3 to 5 ½ yrs and 0 to 4, respectively. The animals were kept on pasture supplemented with palm kernel cake (PKC) at the rate of 1½ kg per animal per day and water was provided *ad libitum*.

Sample collection and hormone assay: Both blood and matched fecal sampling was undertaken at the same time twice per week at 3 to 4 days interval for 9 weeks. Blood was withdrawn from jugular vein into heparinized vacutainer tubes. Collected blood samples were centrifuged at 2500 rpm for 15 min and separated plasma samples were stored at -20°C pending analysis. Fecal samples were collected directly from the rectum using gloved hands and then frozen at -20°C until extraction.

The extraction of fecal steroids was made following the procedure described by Masunda *et al*. (1999) with modifications. Briefly, about 10g of wet feces was dried in an oven at 65°C for 24 hours and then pulverized using an electrical blender (Jakada, Japan). About 0.25g of the ground fecal sample was mixed with 2 mL distilled water, followed by addition of 7 mL diethyl ether and shook once for 30 min. Then, the samples were placed in a freezer (-29°C) for 30 min. The organic component that remained liquid was transferred to another tube and evaporated by placing in a water bath (40°C) under a fume chamber for 50 - 60 min. The extract was then reconstituted by addition of 1 mL methanol and stored at -29°C until analysis.

To determine the concentrations of plasma P_4 and extracted fecal progestins, a commercial solid phase P_4 radioimmunoassay kit (ACTIVE® Progesterone RIA, DSL-3900, USA) was used according to the manufacturer's procedure. Due to the limitation that we had, to get enough RIA kits to analyze all samples collected, the total number of plasma and fecal sample extracts analyzed were 131 each. This comprises all the samples collected from two randomly selected cows (2x18 = 36), the first 10 samples of 5 other cows (5x10 = 50) and the first 9 samples of the remaining 5 cows (5x9 = 45) collected during the first 5 weeks of sampling. The extracted fecal samples were diluted 10 times using an assay buffer (0.01M PBS) before RIA. According to the manufacturer, the sensitivity of the RIA kit for plasma P_4 was 0.12 ng/mL. The intra-assay and inter-assay coeffi-cients of variation for plasma P_4 and fecal progestins analyses as generated by the computer program of the gamma radiation counter were 3.9 and 4.4%, and 2.1 and 3.2%, respectively.

Separation of fecal extracts using HPLC and fraction analysis with RIA: Progesterone metabolites in the fecal extracts were separated by a reverse phase HPLC system, using a CrestPak C18S column (4.6 x 150 mm, Jascoparts Center, Japan) and an isocratic solvent system (mobile phase) of HPLC grade acetonitrile (ACN) in distilled water (40/60, v/v) at a flow rate of 1 mL/min with ultraviolet detection method (Heistermann *et al*., 1998). Extracts of six randomly chosen fecal samples corresponding to the mid-luteal phases of estrous cycle identified based on the plasma P_4 profile were filtered through a 0.45 µm nylon membrane syringe filter (Sun Sri, USA) and then a 20 µL of the samples were injected manually into the HPLC for separation. Fractions of 1 mL/min were separately collected over a period of 60 minutes for progestins. The fractions collected at each minute from three separate HPLC runs were then pooled together to obtain a total of 60 fractions with a volume of 3 mL each. To determine the presence of immunoreactive progestins in the fractions against P_4 antibodies, 25 µL of each of the pooled fractions collected were subjected to the same solid phase P_4 RIA used above. A P_4 reference standard (International Laboratory, USA) was run separately under the same HPLC conditions to determine its peak elution position and retention time.

Data analyses: Data were presented as Mean ± SE. As the data were not normally distributed, Spearman's correlation coefficient (r) was used to test the correlation between the matched plasma P_4 and fecal progestin concentrations at significance level α=0.01 using a statistical package, SPSS v.17.

RESULTS

Correlation between matched fecal progestins and plasma P_4 concentrations: The modified fecal extraction procedure used in this study had a mean recovery efficiency of 76.8±11.8%. A representative graph showing the excretion pattern of fecal progestins in relation to matched plasma P_4 concentrations during the estrous cycle is shown in Fig. 1. There was a significant positive correlation between the concentrations of plasma P_4 and fecal progestins ($r= 0.6$, $P< 0.01$, $n=131$), as tested for the whole group except for one animal that did not show a positive correlation. The follicular phase (FP) and luteal phase (LP) of the estrous cycle were identified based on the plasma P_4 profile, whereby a plasma P_4 concentration of >1 ng/mL was indicative of LP and a P_4 concentration of ≤1 ng/mL as FP (Fig. 1). Comparison of concentrations

of fecal progestins between the FP and LP are shown in Table 1. There was a highly significant difference (P<0.01) in the mean concentrations of progestins between the FP (212.6±19.3 ng/g, n=53) and the LP (792.4±66.7 ng/g, n=78). The concentrations of progestins in the fecal samples ranged from 25.2 to 654.5 ng/g and 53.3 to 2387 ng/g during the FP and LPs, respectively (Table 1). Considering a fecal progestin concentration of 344 ng/g as the cut-off value between the FP and LP, 87% (46/53) of concomitant fecal samples with the FP had a fecal progestin concentration <344 ng/g. In contrast, 77% (60/78) of the fecal samples matched with the LP had fecal progestin concentrations >344 ng/g. Fig. 2 shows the cow that demonstrated a fecal progestins pattern, which was not positively correlated with plasma P_4 pattern.

Radioimmunoassay of HPLC fractions: According to HPLC analysis, there were 3 major peaks of metabolites detected at a retention time of 21.9, 39.5 and 49.3 min. Compared to the other peaks, the one at 21.9 min was closer in elution position but not co-chromatographed with P_4 standard which was eluted at 20.9 min (data not shown). Subsequent analyses of each of the 60 HPLC fractions of fecal extracts (as stated in the methodology) by RIA generated immunoreactive profiles that showed presence of 4 immunoreactive peaks of progestins against the P_4 antibodies corresponding to fraction numbers of 19 to 22, 31 to 34, 47 to 49 and 51 to 54 (Fig. 3).

Table 1: Comparison between LP and FP fecal progestin concentrations of KK cows

Phase of estrous cycle	No. of fecal samples (n)	Fecal progestin (ng/g)	Range (ng/g)
Luteal phase	78	792.4±66.7[a]	53-2387
Follicular phase	53	212.6±19.3[b]	25-655

Values (Mean±SE) with different superscripts along the column show significant difference (P<0.01).

DISCUSSION

The recovery rate obtained for the modified fecal extraction procedure in this study lies within the range (70-100%) provided by several extraction protocols described for ungulates (Schwarzenberger *et al*., 1996b; Heistermann *et al*., 1998; Isobe *et al*., 2005b; Palme, 2005; Capezzuto *et al*., 2008). The slight reduction in the recovery rate compared to Masunda *et al*. (1999; 2002) procedure, however, can be attributed to the decreased amount of diethyl ether and shortened extraction steps. Nevertheless, it is apparent that the current modified procedure is capable to extract quantifiable amount of fecal progestins, at a relatively shorter time and using less amount of solvent.

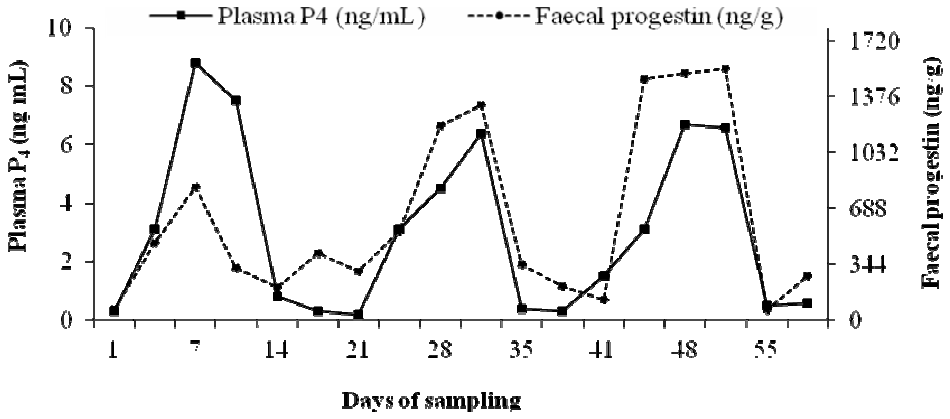

Fig. 1: A representative fecal progestin and plasma P_4 profiles of a cycling KK cow with a significant positive correlation coefficient (r= 0.66, P<0.01).

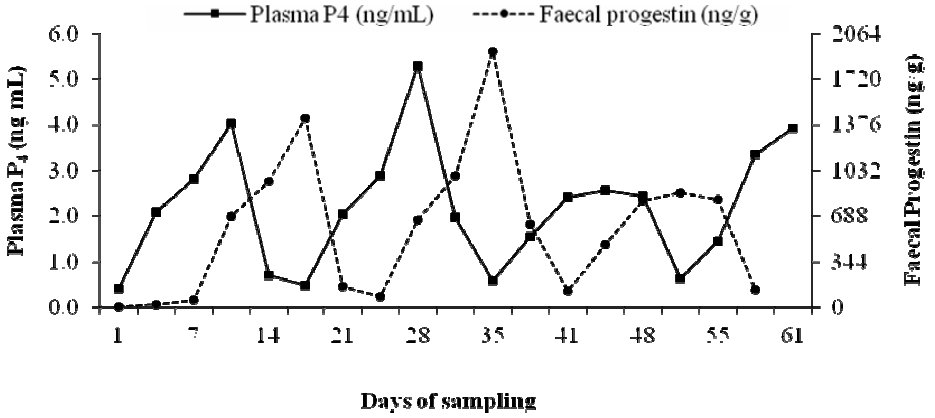

Fig. 2: Plasma P_4 and matched fecal progestin profile during the estrous cycle of a KK cow that didn't show positive correlation. For majority of the fecal samples, the concentration of fecal progestin remains below 344 ng/g, while the plasma P_4 level is >1 ng/mL indicating the LP, and *vice versa*.

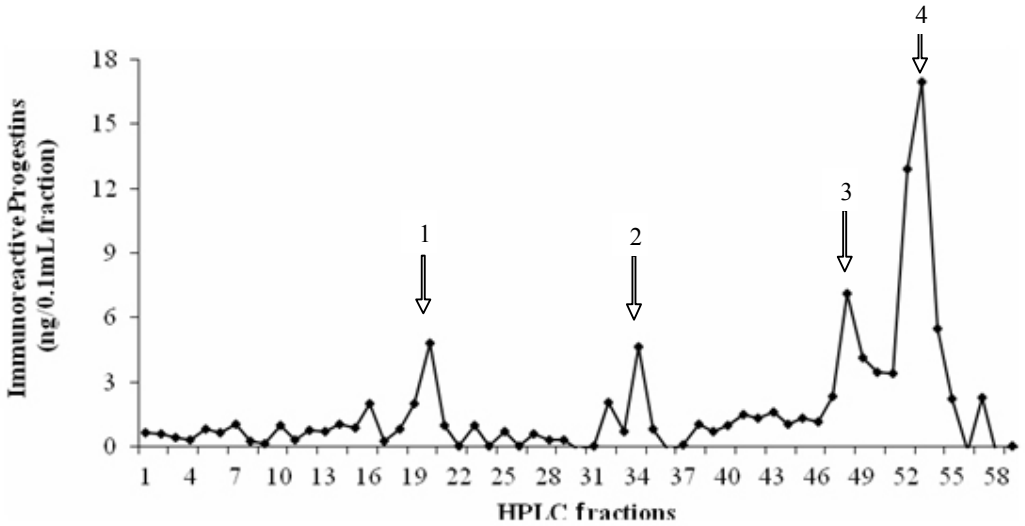

Fig. 3: HPLC profiles of P_4 immunoreactivity in a fecal extract of mid-LP samples of KK cows. Arrows with numbers indicate the elusion position of immunoreactive peaks of fecal progestins. The height of the peaks reflects the degree of immunoreactivity of the metabolites with P_4 antibody of the assay.

The recovery of quantifiable amount of fecal progestins in KK cows and the positive correlation between fecal progestins and plasma P_4 levels are in agreement with previous studies in various breeds of cattle (Palme *et al*., 1996; Schwarzenberger *et al*., 1996b; Masunda *et al*., 1999; 2002; Isobe *et al*., 2005a; 2005b). Significant difference ($P<0.01$) in the mean concentrations of progestins between the FP and the LP fecal samples implies that the assay technique is capable of differentiating the various phases of the estrous cycle. Significant positive correlation ($r=0.6$, $P<0.01$) between the concentrations of fecal progestins and plasma P_4 that reflects the relevance of the changes in fecal progestins to the ovarian events was an evidence for the physiological validity of the use of fecal progestin analysis with RIA to monitor estrous cycle in KK cows. In agreement with this, commercially available antibodies advertised as hormone specific like progesterone have also been used with success for fecal steroid analysis in other breeds of cattle and wildlife (Masunda *et al*., 1999; 2002; Isobe *et al*., 2005a; 2005b; Schwarzenberger, 2007), which was evaluated based on the demonstration of fecal steroid metabolites as reliable indicators of gonadal and adrenal activity (Dehnhard *et al*., 2008).

In this study, there was one cow that demonstrated a pattern of fecal progestins which was not positively correlated with plasma P_4 profile. However, the pattern of fecal progestin profile, when considered irrespective of the plasma P_4 profile, reflected the presence of regular ovarian cycle in that cow. The lack of parallelism between the two hormone profiles in that cow can be attributed to a prolonged time delay of metabolism and excretion of fecal progestin due to impaired liver function and/or prolonged gut passage time. The lag time for excretion of steroids in feces was reported to be affected by several factors and may even vary among individuals of the same species, depending on activity rhythms of individual animals (Palme, 2005), diet (Adams *et al*., 1994; Dantzer *et al*., 2011), and gastro-intestinal problems like constipation (Palme *et al*., 1996). As the animals involved in the present study were all on pasture grazing, dietary interaction with fecal progestin excretion is not expected; however individual animal feed intake may differ and contribute to the extended delay of excretion observed in one cow.

The HPLC analysis of extracts of mid-LP fecal samples in the present study has revealed 3 major metabolite peaks. None of the metabolites detected however, had the same elution position with the authentic P_4 and their specific identification was not possible due to lack of availability of reference standards. According to Schwarzenberger *et al*. (1996b) and other reports (Palme *et al*., 1997; Capezzuto *et al*., 2008), un-metabolized P_4 hormone is barely present in the feces as it is extensively metabolized by the liver and the gut into several 5α and 5β reduced pregnanes (Möstl and Palme, 2002; Palme, 2005; Schwarzenberger, 2007; Adachi *et al*., 2010). This is in agreement with the current finding, in which the HPLC analysis of fecal extracts in KK cows did not show the presence of intact P_4. In contrast, there was a report that indicated the presence of intact P_4 in the feces of Japanese Black beef heifers and cows (Isobe *et al*., 2005a). The variation in the reports on the detection of native P_4 in the feces might be attributed mainly to differences in the extraction procedures, species/breed and to a lesser extent, in the immunoassay methods used (Isobe *et al*., 2005b; Palme, 2005).

Although native P_4 was not detected in the feces of KK cows, measureable amount of fecal progestins were analysed by P_4 antibody RIA to characterise the estrous cycle. This indicates the non-specificity and cross-reactivity of the P_4 antibody of the RIA against the metabolites detected. This was confirmed by the analyses of the 60 HPLC fractions by RIA and establishment of immunoreactive profiles that showed presence of four immunoreactive peaks against the P_4 antibody. This provides evidence for the P_4 RIA used that it was actually tracing relevant metabolites in the fecal extracts during the assay. High degree of cross-reactivity by P_4 antibody which was related to peak heights was observed for the last two fractions, 51-54 and 47-49 together representing

the majority of immunoreactivity, but to a lesser extent for metabolites at fractions 19-22 and 32-34.

The analysis of fecal P_4 metabolites has been used as an appropriate method for monitoring ovarian function and detecting pregnancy in other breeds of cattle using P_4 specific antibody based commercial RIA kits designed primarily to measure P_4 in plasma or serum (Masunda *et al*., 1999; 2002) and also other species of animals using group specific antibodies (Schwarzenberger *et al*., 1996b). Other studies on metabolism based on infusion of radioactively labeled P_4, HPLC separation and subsequent immunoassay of fractions have shown the presence of usually not single, but group of P_4 metabolites in the feces (Schwarzenberger *et al*.,1996b; Adachi *et al*., 2010). The cross-reactivity of the P_4 antibody used in RIA or EIA against the P_4 metabolites found in feces has been attributed to the presence of a common C_{20} carbon atom (20-oxo-pregnanes) in each of the metabolites with the parent hormone P_4 (Schwarzenberger *et al*., 1996a).

Conclusion: The present study demonstrated the physiological validity and potential application of a commercial P_4 antibody RIA to measure fecal progestins for monitoring ovarian cycle in KK cows. The method of extraction and assay used may have also similar role to other wild and domestic ruminants alternative to the invasive method of blood collection for P_4 assay. Although the current extraction method was capable of recovering measureable quantities of progestins at relatively shorter time and using less amount of solvent, both the extraction and the assay method need prior evaluation before any attempt to apply it to other hormones like estrogens. The study has also uncovered the presence of four major immunoreactive metabolites against the P_4 antibody RIA but no native P_4. Further studies using techniques such as gas chromatography-mass spectrometry should be able to characterise and identify the type of immunoreactive metabolites detected.

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