Correlation between plasma progesterone concentrations and fecal Progestins during the estrus cycle of Kedah Kelantan cows[1]

N. Yimer, Y. Rosnina,[2] H. Wahid, A. A. Saharee, K. C. Yap, P. Ganesamurthi, M. Fahmi, M.M. Bukar

Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine,
Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ABSTRACT: The aim of the present study was to determine the correlation between circulating plasma progesterone (P_4) levels and feacal progestins during the estrus cycle of Kedah Kelantan (KK) cows. A total of 12 KK cows pre-synchronized for estrus were subjected to blood and matched fecal sampling twice a week for about 3 cycles. The concentration of progesterone in the plasma and progestins in the fecal samples were determined by using a commercial progesterone radioimmunoassay (RIA) kit (*ACTIVE® Progesterone RIA, DSL-3900*, *USA*). A significant positive correlation between the concentrations of fecal progestins and plasma P_4 was observed ($r = 0.66$, $p<0.001$), as tested for the whole group with the exception of one animal. This implies that the non-invasive measure of fecal progestins using a DSL-3900 RIA kit can be used to monitor the ovarian activity of KK cows. Furthermore, this technique might have also a potential application in monitoring the reproductive activity of captive and free ranging wild life ruminants.

Key words: plasma, fecal, progesterone, progestin, ovarian cycle, KK cows

INTRODUCTION

Reproductive success is controlled mainly by hormones. Some of the important hormones that regulate female reproductive behavior and functions across vertebrates include progesterone and estradiole which are produced by the ovaries (Denhard et al., 2008). Analyses of these hormones are used to validate the reproductive activity of animals (Denhard et al., 2008). The information obtained can be used to improve genetics and reproductive performance (Capezzuto et al., 2008) by increasing the understanding of reproductive cycling and breeding behavior, improvement of estrus synchronization and induction protocols for successful artificial insemination (AI) as well as treating infertility (Penfold et al., 2005; Asa et al., 2006; Graham et al., 2006; Denhard et al., 2008).

The measurement of steroid hormones traditionally involves invasive techniques including blood collection (Capezzuto et al., 2008). Accurate investigation requires the collection of repeated samples, which can be rather stressful even for docile domestic animals (Capezzuto et al., 2008). Moreover, for studies that involve the hypothalamo-pituitary-adrenal (HPA) axis, as the adrenal glands are capable of producing steroids, the results can be confounded by the activation the HPA axis due to the stress caused by catching and restraining before blood sampling, together with venu-puncture (Denhard et al., 2008). Apart from blood sampling, other body fluids such as milk and urine can be collected from animals noninvasively to determine concentration of steroid hormones. Milk sample has been effectively used for progesterone hormone analysis by various studies in cattle (Schwarzenberger et al., 1996); however it is dependent on the physiological status of the animal which thus is restricted to lactating cows and can't be used in heifers and dry cows. Although urine samples have been used successfully to monitor ovarian functions in some domestic animal species (Evans et al., 1984), sampling necessitates restraining the animal in a metabolic cage or following it around to collect midflow samples during urination (Masunda et al., 1999).

To avoid drawbacks associated with invasive blood sampling as well as milk and urine samples for measurement of steroids, a noninvasive method by fecal sampling has been developed in the last

[1] The authors would like to thank Dr Baljit Singh (farm manager) and his staff at the University Agricultural Park for their cooperation and technical assistance during the study period.
[2] Corresponding author: rosnina@vet.upm.edu.my

decade to measure steroids. Analysis of hormones using fecal samples is preferred to using blood samples in both wild and domestic animals for several reasons. For stress-related hormones such as norepinephrine and cortisol, stress during blood collection may increase their values in the plasma. Therefore, feces are of interest as sample material for studying the stress level of animals (Palme et al., 1996). In addition, blood sampling requires facilities for rapid separation (centrifuge) and refrigeration of plasma to avoid the rapid decline of some of the steroid hormones and also requires animals to be restrained very well which makes difficult to conduct studies on free ranging animals (Masunda et al., 1999).

The non-invasive measure of steroids from fecal sample has been applied in various species of animals for various purposes related to reproduction such as, existence of correlation between the changes in fecal steroid concentration with those in the blood, characterizing estrus cycle, monitoring ovarian function, seasonality, pregnancy diagnosis and assessment of various fertility control techniques particularly in free ranging and captive wildlife (Graham, 2004). In domestic ruminants, using non-invasive measure of fecal progesterone metabolites, presence of a strong correlation between the change in fecal concentration and plasma level in goats (Hirata and Mori, 1995; Capezzuto et al., 2008), in ewes (Adams et al., 1994; Palme et al., 1996), and in cows (Masunda et al., 1999; 2002; Isobe et al., 2005) have been reported. Several of these studies however followed various methods of extraction and immunoassay techniques to determine concentrations of steroids. Despite enormous progress of using fecal hormone analysis for research on reproductive biology, each analytical method needs to be validated each time for a particular animal species and hormones, as steroid metabolism by the liver generates vast number of fecal steroid metabolites, yet different in even closely related species (Palme et al., 1996; Heistermann et al., 2006; Denhard et al., 2008).

In this study, a commercially available RIA kit was used to determine the concentration of fecal progestin hormones and to correlate with the circulating plasma concentration of progesterone during the estrus cycle of Kedah Kelantan cows.

MATERIALS AND METHODS

Animals

Kedah Kelantan cows are indigenous beef cattle breed of Malaysia. The study was carried out at the University's dairy and beef farm during the period, May 2009 to July 2009. A total of 12 animals (9 cows all beyond 60 days post partum and 3 heifers), presynchronized for estrus using a synthetic prostaglandin hormone, estrumate (2ml per animal) given intramuscular 11 days apart, were used for the study. The age and parity ranges from 3 to 5 ½ yrs and 0 to 4, respectively. The animals were kept on pasture grazing with palm kernel cake (PKC) supplement (1½ kg per animal per day). Bulls remained separated from cows during the study period.

Sample Collection and Processing

On the same day of the second injection of estrumate for synchronization, both blood and matched fecal sampling were started and continued twice per week at 3 to 4 days interval for about 3 months. Blood was drawn from the jugular vein into a heparinized vacutainer tube. Collected blood samples were then centrifuged (2500 rpm for 15 minutes) and separated plasma samples were stored at -29 ^{o}C pending analysis. Fecal samples on the other hand were collected directly from the rectum using gloved hands. The fecal samples were then frozen at -20 ^{o}C until extraction. The extraction of fecal steroids was made following the procedure described by Masunda et al., (1999), with modifications. Briefly, about 10gm of wet feces was dried in an oven at 65^{o}C for 24 hours and then pulverized using an electrical blender (Jakada, Japan). About 0.25gm of the ground fecal sample was mixed with 2ml distilled water which was followed by addition of 7ml diethyl ether and shaking for 30 minutes. After shaking, the sample was put in a freezer (-29 ^{o}C) for 30 minutes. The organic component that remained liquid was transferred to another tube and let to evaporate by keeping in a water bath (40^{o}C) under a fume chamber. The extract was then reconstituted by addition of 1ml methanol and stored at (-29 ^{o} C) until analysis.

Hormone assay

For the measurement of the concentration of progesterone (P_4) in the blood plasma and progestin in the fecal extract, a commercially available solid phase progesterone radioimmunoassay (RIA) test kit (*ACTIVE® Progesterone RIA, DSL-3900, USA*), was used following assay procedure of the manufacturer. The sensitivity of the assay was 0.12ng/ml. Its intra-assay and inter-assay coefficients of variation were 3.9% and 4.4%, respectively. For the fecal extract, before the assay, the methanol reconstitute was diluted in 1:10 using a standard buffer (PBS, 0.01M).

Statistical analysis

The data was computed using a statistical package, SPSS v.17. As the data were not normally distributed, Spearman's correlation coefficient test (r) was used to test for presence of correlation between plasma P_4 concentration and fecal progestin of matched samples at significance level $\alpha = 0.01$.

RESULTS AND DISCUSSION

A significant positive correlation between the concentrations of P_4 in plasma and progestin in the feces was observed ($r = 0.66$, $P < 0.01$), as tested for the whole group with the exception of one animal that showed a negative correlation. All the animals had regular ovarian cycles but 2 animals in which the correlation was also significantly positive. The concentration of fecal progestin hormone ranges from 61.2ng/g – 413ng/g of feces during the follicular phase and 479.1ng/g – 1552ng/g of feces during the luteal phase.

The non-invasive measure of fecal steroid hormones in cattle has been reported by previous studies (Schwarzenberger et al., 1996; Masunda et al., 1999; 2002; Isobe et al., 2005), although the method of extraction and type of immunoassay used varied. Similar to the present findings, Masunda et al. (1999; 2002) have reported a significant positive correlation between the concentrations of progestins in feces and P_4 in plasma in Zebu cattle breeds using a different RIA kit and following a different extraction procedure. Compared with the extraction method used by Masunda et al. (1999), our modified procedure might be preferred as it shortens the extraction procedure and minimizes the amount of solvent used.

In this study, there was one animal with excretion of fecal progestins negatively correlated with the plasma P_4 which might be due to a prolonged time delay of metabolism and excretion. As steroids are metabolized in the liver and excreted via the bile into the gut, measured amounts of fecal steroids reflects an event of certain time ago (Palme, 2005). Variation in lag time depends on species, and sometimes within species, depending on the activity rhythms of animals. Although it is not possible to know the actual reason from this study for the exceptional delay in excretion observed in this animal, it might be a consequence of impaired liver function and/or prolonged gut passage time. Otherwise, the fecal progestin pattern alone reflects the presence of regular ovarian cycle in the cow, like the plasma P_4 profile. The observed positive correlation between the concentration of fecal progestin and plasma P_4 in two animals with irregular ovarian cycle might imply the potential use of non-invasive measure of fecal progestin in diagnosing reproductive disturbances.

The assay technique used in this study was designed to measure progesterone in human serum or plasma. Hence, it was important for biological validation to be performed to demonstrate that the technique can detect biologically meaningful changes in circulating progesterone concentration. One of the ways by which an assay technique is validated is by comparing and correlating fecal metabolite level with levels of parent hormones in the blood (Palme, 2005; Capezzuto et al., 2008; Denhard, 2008; Heistermann et al., 2008). Therefore, in this study, the observed significant positive correlation between the concentrations of fecal progestins and plasma P_4 confirms the biological validity of the assay technique used providing relevant information that reflects the physiological event in question.

CONCLUSIONS

It is concluded that the non invasive measure of fecal progestin using a commercially available progesterone RIA kit (*ACTIVE® Progesterone RIA, DSL-3900, USA*) might be used as alternative technique to monitor the ovarian activity of Kedah Kelantan Cows. This technique might have also a

potential application in monitoring the reproductive activity of wild life ruminants using KKs as models.

LITERATURE CITED

Adams, N. R., Abordi, J. A., Briegel, J. R., & Sanders, M. R. (1994). Effect of diet on the clearance of estradiol-17 beta in the ewe. Biol Reprod, 51, 668-674.

Asa, C.S., Bauman, K., Callahan, P., Bauman, J., Volkmann, D.H., Jöchle, W. (2006). GnRH agonist induction of fertile estrus with either natural mating or artificial insemination, followed by birth of pups in gray wolves (Canis lupus). Theriogenology, 66, 1778-1782.

Capezzuto, A., Chelini" , M.O.M., Felippe, E.C. G., and Oliveira, C.A. (2008). Correlation between serum and fecal concentrations of reproductive steroids throughout gestation in goats. Animal Reproduction Science, 103, 78-86.

Dehnhard, M., Naidenko, S., Frank, A., Braun, B., Göritz, F., Jewgenow, K. (2008). Non-invasive monitoring of hormones: a tool to improve reproduction in captive breeding of the Eurasian lynx. Reprod Domest Anim., 43, 74-82.

Evans, K.L., Kasman, L.H. and Hughes, J.P. (1984). Pregnancy diagnosis in the domestic horse through urinary estrone conjugate analysis. Theriogenology, 22, 615-620.

Graham, L.H. (2004). Non-Invasive Monitoring of Reproduction in Zoo and Wildlife Species. ARBS Ann Ver Biomed Sci, 6, 91-98.

Graham, L.H., Byers, A.P., Armstrong, D.L., Loskutoff, N.M., Swanson, W.F., Wildt, D.E., Brown, J.L. (2006). Natural and gonadotropin-induced ovarian activity in tigers (Panthera tigris) assessed by fecal steroid analyses. Gen Comp Endocrinol, 147, 362-370.

Heistermann M., Palme R., Ganswindt A. (2006). Comparison of different enzyme immunoassays for assessment of adrenocortical activity in primates based on fecal analysis. Am J Primatol, 68, 257-273.

Hirata, S., & Mori, Y. (1995). Monitoring reproductive status by fecal progesterone analysis in ruminants. J Vet Med Sci, 57(5), 845-850.

Isobe, N., Nakao, T., Yamashiro, H., & Shimada, M. (2005). Enzyme immunoassay of progesterone in the feces from beef cattle to monitor the ovarian cycle. Anim Reprod Sci, 87, 1-10.

Masunda B., Mutisi C., Hamudikwanda H., Agumbah J.G.O. (1999). The concentration of faecal progestins during the oestrus cycle of Nkone cows and the effect of duration of storage of faecal samples at room temperature on faecal progestin levels. Tropical Animal Health and Production. 31, 373-381.

Masunda B., Mutisi C., Hamudikwanda H., Agumbah J.G.O. (2002). The use of faecal progestin measurements to monitor reproductive activity of in Mashona cows in a small holder farming area of Zimbababwe. Tropical Animal Health and Production, 34, 309-318.

Palme, R., 2005. Measuring fecal steroids guideline for practical application. Ann. N. Y. Acad. Sci., 1046, 75–80.

Palme, R., Fischer, P., Schildorfer, H. and Nismail, M. (1996). Excretion of infused 14C-steroid hormones in faeces and urine in domestic livestock. Animal Reproduction Science, 43, 43-63.

Penfold L.M., Monfort S.L., Wolfe B.A., Citino S.B., Wildt D.E. (2005). Reproductive physiology and artificial insemination studies in wild and captive gerenuk (Litocranius walleri walleri). Reprod Fertil Dev, 17, 707–714.

Schwarzenberger, F., M□stl, E., Palme, E., Bamberg, E. (1996). Fecal steroid analysis for non-invasive monitoring of reproductive status in farm, wild and zoo animals. Animal Reproduction Science, 42, 515–526.