UNIVERSITI PUTRA MALAYSIA

PRESERVATION, EXTRACTION AND ANALYTICAL METHODS FOR FAECAL PROGESTERONE METABOLITE IN COWS AND IMMUNE RESPONSE TO PREGNANOLONE HEMISUCCINATE IN RABBITS

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

July 2019

In all your ways acknowledge Him and He shall direct your paths

Proverbs 3:6

DEDICATION

This thesis is dedicated to:

Kwasku Mada
my paternal grandmother: whose prayers for me are never ending

Prof. Peter M. Bzugu and Mrs. Lucy M. Peter
my parents: who have taught me to do the best I can possibly do and it will be enough and also making me believe that everything is possible as long as i have faith in it

Mary Adarju Innocent and Martha Kwasku Innocent
my children: who always tell me 'daddy, hurry up and return home'

Barr. (Mrs.) Munakur Samiyu Peter
my lovely wife: for her unrelenting daily prayers and priceless patience.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Doctor of Philosophy

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Chairman : Abd Wahid Haron, PhD
Faculty : Veterinary Medicine

Monitoring reproductive function using faecal progesterone metabolite evaluations is a well-established technique in gazelles, horses, baboon, pigs, rhinoceros, elephants and should be extensively applied in domestic animals. Some factors however, prevent the application of this non-invasive technique to monitor reproduction in animals. These factors include paucity of practical information on stability of faecal progesterone metabolites under different preservation and extraction methods as well as sensitivity and specificity of analytical techniques. Therefore, general objective of this study was to evaluate preservation, extraction and analytical techniques for progesterone metabolite in faeces of cows; determine plasma progesterone and progesterone metabolite profiles in pregnant and non-pregnant cows; modify existing high performance liquid chromatography (HPLC) method for steroid hormones for the simultaneous detection of multiple progesterone metabolites in faeces of cows and to determine the immune responses and cellular changes following administration of low dose of pregnanolone hemisuccinate in rabbits. Progesterone metabolites were extracted from faecal samples using already established procedures and their concentrations together with plasma progesterone levels were determined using radioimmunoassay. A mobile phase made of distilled water and acetonitrile (70:30 v/v) and a stationary phase made up of C_{18} column coupled to an Agilent HPLC 1100 series module was modified from existing HPLC method of steroid hormones to detect progesterone metabolites in faeces of cows. This modified HPLC method was hypothesized to be able to serve as an alternative to use of immunoassay on the analysis of progesterone metabolite in animals. Male rabbits were parenterally administered with pregnanolone hemisuccinate on day 0 and were given a booster dose on day 14 so as to produce group specific antibody to progesterone metabolites. Antibody response was determined using IgG and IgM evaluations using ELISA. The results show that there is no statistically significant difference in progesterone metabolite concentration in faeces that were dried at 70°C (9.33±5.05 ng/g), 50°C

(5.66±4.19 ng /g) or at 90°C (5.29±2.51 ng/g). Similarly, radioimmunoassay technique was used to determine the stability of progesterone metabolites in oven dried and fresh faecal samples. Faecal progesterone metabolite concentrations were found to be higher when extracted from oven-dried samples (2.06±1.58 ng /g) as compared to extraction from fresh faecal samples (0.49±0.30 ng /g). This difference was found to be statistically significantly ($P<0.05$). When un-preserved faeces were left exposed to environmental conditions for several days, progesterone metabolite concentrations therein were observed to rise from 11.04±7.68 ng/g at 0 hrs to maximum value of 31.71±10.33 ng /g at 48 hrs and thereafter decline to 36.62±12.30 ng/g after 216 hrs. There was no statistically significant difference ($P>0.05$) among these values. The relationship between faecal progesterone metabolites concentration and plasma progesterone concentration in cows were also investigated in this study. The results showed a correlation between plasma progesterone and faecal progesterone metabolites in pregnant ($r=0.211$, $n=8$, $p=0.23$) and in non-pregnant cows ($r=0.209$, $n=8$, $p=0.35$). A High-Performance Liquid Chromatography (HPLC) method for steroid hormones commonly employed in other species of animals was modified and used in this study for cows using 5β-pregnan-3α-ol-20-one and 3β-Hydroxy-5α-pregnan-20-one sulphate pyridine salt as internal standards. UV detection was employed to monitor the eluent from a stationary phase at an excitation wavelength of 254 nm and an emission wavelength of 360 nm. The linearity of the calibration curve was obtained in the concentration range of 500-7500 µg/µL for 5β-pregnan-3α-ol-20-one and 3-15 µg/µL for 3β-Hydroxy-5α-pregnan-20-one sulphate pyridine salt respectively. The co-efficient of determination was found to be 0.9977 for 5β-pregnan-3α-ol-20-one and 0.9776 for 3β-Hydroxy-5α-pregnan-20-one sulphate pyridine salt. The production of group specific antibodies for progesterone metabolites using the immunogen in rabbits was not achieved in this study. This could have been due to minute amount of the immunogen used or a defective bioconjugation method or due to inherent molecular properties of 5β-pregnan-3α-ol-20-one. Histopathological, haematological and biochemical parameters did not significantly differ between control and immunised rabbits in this study. This study provides practical and reliable information on faecal progesterone metabolite levels exposed to different storage, preservation and extraction parameters in cows. Best results for the analysis of faecal progesterone metabolites in cows are obtained when faeces are dried at 70°C and are extracted soon after voiding. The correlation observed between faecal progesterone metabolites and plasma progesterone concentrations shows that faecal progesterone metabolites concentration can be used for determination of reproductive status in cows. The HPLC method described in this study is simple, practical and rapid and can be applied for determination of progesterone metabolite concentration. It is therefore recommended that the information provided by this study be utilised in the non-invasive study of reproductive function in cows. These techniques are simple, reliable and can be performed within a short time. Group specific antibodies were not produced using 5β-pregnan-3α-ol-20-one-hemisuccinate, it is recommended that a modification in the bioconjugation method used or immunisation procedure be performed. Furthermore, other progesterone metabolites could be used to produce group specific antibodies and thereafter used in immunoassays to produce a more specific and sensitive analysis.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagi menenuhi keperluan untuk ijazah Doktor Falsafah

PEMULIHARAAN, PENGEKSTRAKAN DAN KAEDAH ANALISIS METABOLIT PROGESTERON DALAM TINJA LEMBU DAN TINDAK BALAS IMUN TERHADAP PREGNANOLONE HEMISUCCINATE DALAM ARNAB

Oleh

INNOCENT DAMUDU PETER

Julai 2019

Pengerusi : Abd Wahid Haron, PhD
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Pemantauan fungsi pembiakan menggunakan metabolit progesteron tinja adalah teknik yang telah digunakan dalam beberapa spesis haiwan dan seharusnya digunakan secara meluas dalam haiwan domestik. Beberapa faktor menjadi penghalang untuk mengaplikasikan teknik tidak invasif ini dan menyelia pembiakan di kalangan haiwan. Faktor-faktor ini termasuk kekurangan maklumat praktikal mengenai kestabilan metabolit progesteron tinja dari segi kaedah pemeliharaan dan pengekstrakan yang berlainan serta kepekaan dan kekhususan teknik analisis.. Objectif kajian ini adalah: untuk menilai pemeliharaan, pengekstrakan dan teknik analisis metabolit progesteron dalam tinja lembu; menentukan progesteron plasma dan profil progestagen dalam lembu hamil dan tidak hamil; menghasilkan kaedah kromatografi cecair prestasi tinggi (HPLC) yang mudah dari kaedah lama yang telah ada untuk mengesan metabolit progesteron secara serentak dalam bentuk najis lembu dan untuk menentukan tindak balas imun dan perubahan sel selepas pemberian dos rendah ke pregnanolone hemisuccinate dalam arnab. Metabolit progesteron diekstrak daripada sampel tinja menggunakan prosedur yang telah ditetapkan dan kepekatan progesteron plasma dan metabolit ditentukan menggunakan radioimmunoassay. Fasa mudah alih yang diperbuat daripada air sulingan dan asetonitrile (70:30 v / v) dan satu fasa pegun yang terdiri daripada kolum C_{18} dan modul siri Agilent HPLC 1100 untuk menghasilkan kaedah yang baru untuk mengesan metabolit progesteron dalam tinja lembu. Arnab jantan telah digunakan dengan suntikan parenteral pregnanolone hemisuccinate pada hari 0 dan dos penggalak telah disuntik pada hari ke 14 untuk menghasilkan antibodi khas kumpulan untuk metabolit progesteron. Tindak balas antibodi ditentukan menggunakan penilaian IgG dan IgM menggunakan ELISA. Hasilnya menunjukkan kepekatan metabolit progesterone yang lebih tinggi diperoleh apabila tinja dikeringkan pada suhu 70 ° C (9.33 ± 5.86 ng/g) berbanding suhu pengeringan pada suhu 50 ° C (7.37 ± 6.91 ng/g) atau 90 ° C 5.29 ± 2.51 ng/g). Perbezaan ini

bagaimanapun tidak ketara secara statistik (P> 0.05). Teknik radioimmunoassay digunakan untuk menentukan kestabilan metabolit progesteron dalam tinja segar yang dikeringkan dalam ketuhar. Kepekatan metabolit progesteron tinja didapati lebih tinggi apabila diekstrak dari sampel pengeringan oven (3.06 ± 3.12 ng/g) berbanding dengan sampel dari tinja segar (0.49 ± 0.30 ng/g). Perbezaan ini didapati ketara secara statistik (P>0.05). Apabila tinja yang tidak dipulihara terdedah kepada keadaan sekitar, kepekatan metabolit progesterone meningkat dari 29.37 ± 37.07 ng / g pada 0 jam ke nilai maksimum 86.23 ± 73.20 ng / g pada 48 jam dan kemudian menurun ke 46.12 ± 38.04 ng / g selepas 216 jam. Tiada perbezaan ketara secara statistik (P>0.05) di antara nilai-nilai ini. Hubungan antara kepekatan metabolit progesteron tinja dan kepekatan progesteron plasma dalam lembu juga diselidiki dalam kajian ini. Keputusan menunjukkan hubungan antara plasma progesteron dan metabolit progesteron faecal semasa mengandung (r = 0.211, n = 8, p = 0.23) dan dalam lembu tidak hamil (r = 0.209, n = 8, p = 0.35). Kaedah kromatografi cecair prestasi tinggi untuk hormone steroids yang digunakan untuk haiwan species lain telah diubahsuai dengan menggunakan garam pyridine sulfat 5β-pregnan-3α-ol-20-one dan 3β-Hydroxy-5α-pregnan-20-one sebagai piawaian dalaman. Pengesanan UV digunakan untuk memantau bahan pengelusi dari lajur pada panjang gelombang pengujaan 254 nm dan panjang gelombang pelepasan 360 nm. Faktor linear lengkungan yang diperoleh dalam julat kepekatan 500-7500 μg / μL untuk 5β-pregnan-3α-ol-20-one dan 3-15 μg / μL untuk 3β-Hydroxy-5α-pregnan-20-one garam pyridine. Koefisien penentuan didapati 0.9977 untuk 5β-pregnan-3α-ol-20-one dan 0.9776 untuk 3β-Hidroksi-5α-pregnan-20-one garam pyridine sulfat. Pengeluaran antibodi spesifik kumpulan untuk metabolit progesteron menggunakan 5β-pregnan-3α-ol-20-one-hemisuccinate dalam arnab tidak dicapai dalam kajian ini. Ini mungkin disebabkan oleh bilangan kecil imunogen yang digunakan atau kaedah biokonjugasi yang rosak atau disebabkan sifat molekul yang wujud 5β-pregnan-3α-ol-20-one. Histopatologi, hematologic dan parameter biokimia tidak menunjukkan perbezaan ketara antara kawalan danarnab yang telah di imunisasikan dalam kajian ini. Kajian ini memberikan maklumat praktikal dan boleh dipercayai mengenai tahap metabolit progesteron tinja yang terpengaruh dengan pelbagai cara penyimpanan, pemeliharaan dan pengekstrakan dalam lembu. Hasil terbaik untuk analisis metabolit progesteron tinja pada lembu diperoleh apabila ia dikeringkan pada suhu 70 ℃ dan diekstrak secepat mungkin selepas dikeuarkan. Korelasi di antara metabolit progesteron tinja dan konsentrasi progesteron plasma menunjukkan bahawa konsentrasi metabolit tinja boleh digunakan untuk menentukan status pembiakan dalam lembu. Kaedah HPLC yang di terangkan dalam kajian ini adalah mudah, praktikal dan pantas dan boleh digunakan untuk menentukan konsentrasi metabolit progesteron. Oleh yang demikian, maklumat daripada kajian ini boleh digunakan untuk mengkaji fungsi pembiakan lembu secara tidak invasif. Teknik ini mudah, boleh dipercayai dan boleh dilakukan dalam masa yang singkat. Antibodi khas kumpulan tidak dihasilkan menggunakan 5β-pregnan-3α-ol-20-one-hemisuccinate, dan disarankan agar pengubahsuaian biokonjugasi atau prosedur imunisasi dilakukan. Tambahan pula, metabolit progesteron lain boleh digunakan untuk menghasilkan antibodi spesifik kumpulan dan ia digunakan dalam immunoassays untuk menghasilkan analisis yang lebih spesifik dan sensitif.

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This Thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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%	Percentage
µL	Micro litre
µm	Micro meter
^{0}C	Degree centigrade
^{125}I	Iodine
ALT	Alanine amino transferase
ANOVA	Analysis of variance
APC	Antigen presenting cells
AST	Aspartate amino stransferase
AUP	Animal utilisation protocol
BSA	Bovine serum albumin
CFA	Complete Freunds Adjuvant
CBC	Complete blood count
CL	Corpus luteum
cpm	Count per minute
Da	Dalton
DLC	Differential leucocyte count
E_2	Estradiol
EIA	Enzyme immuno assay
ELISA	Enzyme linked immunosorbent assay
Fl	Femto litre
FSH	Follicle stimulating hormone
G	Guage
Glu	Glucose

GnRH	Gonadotrophin releasing hormone
h	Hour
H&E	Hematoxylin and Eosin
H_0	Null hypothesis
H_A	Alternate hypothesis
Hb	Hemaglobin
HPLC	High performance liquid chromatography
IACUC	Institutional animal care and use committee
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
KDA	Kilo Dalton
KLH	Keyhole Limpet Hemolysin
KPa	Kilo pascal
LC	Liquid chromatography
LOD	Limit of detection
LLOQ	Lower limit of quantification
LH	Luteinizing hormone
M	Molar
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
mg	Milligram
MHC	Major histocompatibility complex

mL	Milli litre
n	Number
ng	Nanogram
nm	Nano metre
p	Statistical significance level
PBS	Phosphate buffered saline
PCV	Packed cell volume
$PGF_{2\alpha}$	Prostaglandin$F_{2\alpha}$
pH	Potential of Hydrogen
PLT	Platelets
r^2	Coefficient of determination of linear regression
RBC	Red blood cells
RIA	Radio immunoassay
Rt	Retention time
SPSS	Statistical package for social scientist
Th	Helper T cells
TP	Total Protein
UV	Ultra violet
v/v	Volume per volume
WBC	White blood cells

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Progesterone, a steroid hormone made up of 21 carbon atoms, is produced by granulosa-lutein cells located within corpus luteum of the ovary. The hormone is released into the circulatory system soon after synthesis where it is transported principally to the uterus (Al-Asmakh, 2007; Barros et al., 2015). Progesterone plays a fundamental role in reproductive events in animals as it is directly associated with events leading to the establishment and maintenance of pregnancy such as ovulation and implantation (Conneely et al., 2002; Heyman et al., 2002; Al-Asmakh, 2007; Kornmatitsuk et al., 2007; Capezzuto et al., 2008; Carter et al., 2008). During pregnancy, additional amounts of progesterone is produced by the syncytiotrophoblast of the placenta and by the *zona fasciculata* and *zona reticularis* of the adrenal cortex (Adashi et al., 1996; Al-Asmakh, 2007; Barros et al., 2015). Plasma progesterone concentrations are often used as a reflection of ovarian function in animals and have been used to classify oestrus cycle into two distinct phases: the luteal (periods of progesterone dominance in plasma) and follicular (periods of declining plasma progesterone) phases (Kornmatitsuk et al., 2007). During luteal phase, progesterone is known to actively cause endometrial cellular proliferation in animals in preparation to implantation following an eventual mating and subsequent fertilisation (Al-Asmakh, 2007). Plasma progesterone levels remain elevated during luteal phase and pregnancy. This elevated progesterone levels have been used to diagnose pregnancy or monitor oestrous cycle in animals (Masunda et al., 1999; Carter et al., 2008).

For a successful determination of ovarian function in animals, steroid hormones such as progesterone are evaluated from serially collected blood samples (Nugraha et al., 2017). However, the repeated collection of blood samples is not always possible in animals (Schwarzenberger et al., 1997). Non-invasive samples such as hair, urine, milk and faeces are reported to have quantifiable amount of steroid hormone that could serve diagnostic purposes (Kumar et al., 2013). However, there are some limitations in utilising such non-invasive samples for monitoring reproductive function in animals. Hair samples are only suitable for determination of long-term hormone levels in animals and are not suitable for short term evaluation of hormone levels (Ventrella et al., 2018). Collection of urine samples in animal will require animal restraint for insertion of catheters and this eventually limit the use of this type of sample (Kumar et al., 2013). Milk samples on the other hand are limited to lactating animals and cannot be used for heifers or dry-off animals (Kumar et al., 2013). Furthermore, animal handling and restraint to obtain samples may potentially because capture induced-stress and could affect results of hormone analysis. Faecal samples however can be collected repeatedly in animals with ease and without causing any danger, pain or distress to the subject (Gholib et al., 2017). Furthermore, progesterone metabolites are mainly excreted through the faeces of animals (Schwarzenberger et al., 1996; Kumar et al., 2013; Gholib et al., 2017). Biological samples collected without restraint

provide precise amounts of faecal steroid hormones. These advantages therefore make faeces to be a practical choice for repeated sample collection for analysing steroid hormones for monitoring reproductive function in animals (Schwarzenberger et al., 1996; Ziegler and Wittwer, 2005).

The terms "faecal progesterone", "progestogens", "progestins" and "progestagens" have been used rather loosely in literature when referring to progesterone metabolites in faeces of animals (Van der Goot et al., 2015; Mithileshwari et al., 2016; Cao et al., 2016; Davis, 2017; Gascoigne et al., 2017; Silvestre et al., 2017). The term progesterone is strictly reserved for a natural hormone that is synthesized and released by the corpus luteum in the ovary. Since natural progesterone is not present in feces, it becomes proper that the term faecal progesterone metabolites be used (Schwarzenberger et al.,1997). Progestogens refers to a group of naturally occurring progesterone metabolites while progestins is used to describe synthetic progestational agents that are designed to target and bind to progesterone receptors (Sitruk-Ware, 2008). The term "progestagens" appears to be a better terminology for faecal progesterone metabolites as the extent to which faecal progesterone metabolite possess progestational activity is not fully understood.

The half-life of progesterone is reported to be approximately 5 minutes, after which the hormone is extensively metabolised into several metabolites by the liver (Schumacher and Robert, 2002; Al-Asmakh, 2007). Progesterone is first converted into 5α-dihydro-progesterone by cytochromes P450 enzyme (Shimada et al., 1994) and is thereafter metabolised by the liver to yield pregnanolone and several other metabolites before their eventual excretion in faeces (Lemoine et al., 1993; Shimada et al., 1994; Schwarzenberger et al., 1997). Earlier studies on progesterone metabolites were performed to elucidate on the metabolism and route of excretion of natural progesterone. Such studies have now paved ways for further research that have now demonstrated the application of faecal progesterone analysis to determine reproductive function in animals (Hirata and Mori, 1995; Capezzuto et al., 2008). Data from several studies have established that faecal progesterone metabolites concentrations exhibit a similar pattern to plasma progesterone in animals (Schwarzenberger et al., 1996; 1997; Masunda et al., 1999; Capezzuto et al., 2008; Yimer et al., 2012). By using immunoassay, several studies have described a positive correlation between plasma progesterone and faecal progesterone metabolite concentrations. This has led several researchers to utilise faecal progesterone metabolites evaluations for characterizing reproductive status of animals (Desaulniers et al., 1989; Schwarzenberger et al., 1992; Masunda et al., 1999; Graham et al., 2001; Isobe et al., 2005a; Gan and Patel, 2013). Faecal progesterone metabolite evaluations have now been successfully used to determine the onset of puberty, corpus luteum function, pregnancy, abortion and in monitoring therapy of the reproductive system in an ever-expanding number of animal species (Desaulniers et al., 1989; Hirata and Mori, 1995; Borjesson et al., 1996; Wasser et al., 1996; Masunda et al., 1999; Pereira et al., 2006; Ncube et al., 2011).

Faecal steroid analysis is a potent tool that can provide information on physiology and reproductive status in a wide range of animal species (Capezzuto et al., 2008). The use of faecal progesterone metabolites for monitoring reproductive function in free ranging animals have been extensively applied in free ranging terrestrial animals such as rhinoceros (Schwarzenberger et al., 2000), cheetah (Terio et a;., 2002), lion (French et al., 2003), boar (Macchi et al., 2010), zebra (Ncube et al., 2011), panda (Deng et al., 2014), deer (Mithileshwari et al., 2016; Wang et al., 2016), tiger (Panda et al., 2017), jaguar (Conforti et al., 2017), gazelles (Wojtusik et al., 2017), parrots (Pereira et al., 2018) and even sea animals such as whales (Larson et al., 2003) and otters (Rolland et al., 2005). In these animal species, many techniques for analysing progesterone metabolites have been established and are routinely used. Generally, fresh faecal samples are used in analysis of faecal progesterone metabolites in terrestrial animals. In aquatic animals however, drying of faecal samples usually precede analysis of faecal progesterone. Although analysis of progesterone metabolites in terrestrial animals is done using fresh faecal samples, there is little information on the concentration of faecal progesterone metabolite in fresh and dried faecal samples in domestic ruminants. Studies are required to elucidate on the value of using fresh faecal samples for analysis progesterone metabolites.

Despite the widespread application of non-invasive techniques for monitoring reproduction in many animal species, faecal progesterone metabolites analysis has not been extensively applied to monitor reproductive function in farm animals. This could partly be due to a failure in establishing newer analytical techniques for animals. Use of non-invasive samples for quantifying faecal steroid hormone could greatly improve animal welfare. The development and validation of a new analytical method is the first step before adopting any method in a defined animal species. Traditionally, immunoassay systems using antibodies to progesterone are used to determine the total concentration of progesterone metabolite in faeces of animals. This is due to the widespread availability of antibodies to natural progesterone and because such antibodies have abilities to cross react with progesterone metabolite molecules in feces. Due to this reason, progesterone metabolites are easily quantified. Antibodies against any of several progesterone metabolites have not been produced commercially. The development of immunoassay systems with antibodies to progesterone metabolites would have more specificity and sensitivity as compared to the more readily available immunoassay systems made with antibodies to progesterone. Among several factors to be considered for production of antibodies is immunogen preparation as most steroid hormone metabolites have low molecular weight and are non-immunogenic on their own. High Performance Liquid Chromatography (HPLC) methods for progesterone have been previously described in literature for steroid hormone and their metabolites. HPLC methods for progesterone metabolites using non-faecal samples are earlier being described. Newer and highly specific methods for detection of individual faecal progesterone metabolites therefore become necessary which will improve the methodologies employed in non-invasive methods of monitoring reproductive function in animals.

To fully utilize the faecal progesterone metabolite analysis in farm animals for monitoring reproductive function, optimum preservation, extraction and storage conditions needs to be determined and evaluated (Pappano et al., 2010). One important challenge in the analysis of faecal steroids is the preservation and storage of faecal samples before analysis (Nugraha et al., 2017). Faecal bacterial enzymes have been reported to alter the composition and concentration of steroids hormone metabolite when faeces are left unpreserved (Khan et al., 2002; Nugraha et al., 2017). Post defecation changes in progesterone concentration in faeces of animals have been reported. This has been mainly attributed to the activity of faecal bacteria or bacterial enzymes (Kumar et al., 2013). In cows, there is paucity of information on the fate of progesterone metabolite in voided faeces over time. This therefore necessitate the preservation of faecal samples in preservatives or storage at sub-zero temperatures before analysis is indicated (Ziegler et al., 2000; Nugraha et al., 2017). This is however not always achievable (Khan et al., 2002). Drying of faecal samples using oven or using solar box cooker are alternatives to freezing (Galama et al., 2004). Other alternative methods are storage in ethanol or sodium azide and kept at room temperature (Khan et al., 2002). Several studies have explained the degradative activities of faecal bacteria on progesterone metabolites after voiding (Schwarzenberger et al., 1996; Kumar et al., 2013). However, the short- and long-term degradative effects of faecal bacteria on progesterone metabolites in cow faeces remains to be fully understood. This information may allow an informed decision as to how long can voided faeces from cows be of analytical use.

1.2 Problem statement

The assessment of reproductive function is traditionally performed using blood samples. Blood sample collection in animals requires restraint which is associated with both pain and stress. Animal restraint procedures affects hormone concentration in animals and could give inaccurate results. Because of this, blood sample analysis is now fast giving way to non-invasive sample analysis such as faeces in animals to determine steroid hormone levels. Within the last few decades, there has been an increasing interest in the use of non-invasive faecal steroid analytical methods for monitoring reproduction in animals just as concerns about animal welfare grows. However, before adopting a new non-invasive faecal steroid methodology in a new species, the preservation, extraction and analytical methods for faecal steroids need to be assessed. This is because several methodologies for preservation, extraction and storage methods are available and have been established in some animal species. Equally, multiple types of HPLC and immunoassay methods are utilised in the determination of total progesterone metabolites in biological samples. HPLC methods and immunoassay system determines the concentration of individual progesterone metabolite in faecal samples. Commercially available immunoassay systems prepared with antibodies to natural progesterone are acceptable immune assay systems for quantifying faecal progesterone metabolites. These kits have significant cross reactivity with many progesterone metabolites and are thus rightly used. However, production of antibodies against pregnanolone as a group specific antibody would greatly provide higher analytical precision for a better analytical result in future.

1.3 Justification of the study

The determination of reproductive status of animals by faecal steroid analysis is one of valuable tools for efficient management of animals. This technique is safe, non-invasive, and simple and could be used as an alternative to invasive monitoring of reproductive function in animals. Faecal progesterone metabolite analysis is therefore explored in this study as a non-invasive tool to monitor reproductive status for domestic animals.

1.4 Research hypotheses

The general hypothesis for this study is that under appropriate preservation and extraction methods, faecal progesterone metabolite evaluations can be used to monitor the oestrous cycle and pregnancy in cows.

1.5 General objectives

The general objective of this study is to evaluate and determine best preservation and extraction methods for progesterone metabolites in faeces of cows. The study is also designed to determine immune response towards pregnanolone hemisuccinate using an animal model (rabbit).

1.5.1 Specific objectives

The specific objectives of this study are to:

1. evaluate preservation and extraction methods for progesterone metabolites in faeces of cows
    i. determine the optimum oven drying temperature for faeces that would be used for progesterone metabolites analysis in cows
    ii. to determine the post defecation changes in progesterone metabolite levels in unpreserved feces
    iii compare the concentrations of faecal progesterone metabolites concentrations in fresh and oven-dried faecal samples
2. determine the relationship between plasma progesterone concentration and progesterone metabolite concentration in faeces of pregnant and non-pregnant cows.
3. apply a high-performance liquid chromatography method for the detection of progesterone metabolites in faeces of cows.
    i. demonstrate the application of this method for the analysis of progesterone metabolites in faecal samples.
4. determine immune response as well as haematological, biochemical and histopathological changes following parenteral administration of pregnanolone hemisuccinate in rabbits.

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The student, Innocent Damudu Peter was born on 25th January, 1985 in Madagali, Madagali Local Government Area, Adamawa State, Nigeria. He did his primary education in Kings Private School, Mairi Ward, in Maiduguri Borno State between 1990-1996. He then joined Government College Maiduguri for his Secondary School in 1997 and graduated in 2001. In 2002 he got admission to study Veterinary Medicine in the Faculty of Veterinary Medicine, University of Maiduguri and graduated with a DVM degree in the year 2009. After completion of his University education, he then went to Calabar and served as a resident clinician at the State Veterinary Clinic, Calabar for his mandatory national youth service. After finishing his national service, he enrolled for the Master of Veterinary Science in Theriogenology at the school of post graduate studies, University of Maiduguri, Maiduguri, Nigeria. During his post graduate studies, he got employed as an assistant lecturer in the Department of Veterinary Surgery and Theriogenology, University of Maiduguri in 2011. In April 2016, he enrolled for a Doctor of Philosophy (PhD) degree in the field of Theriogenology under the supervision of Prof. Abd. Wahid Haron at the Department of Clinical Studies, Faculty of Veterinary Medicine, Universiti Putra Malaysia. He has attended many Veterinary congresses, conferences and seminars. He has also published many articles in peer reviewed journals. Publications arising from his doctoral research are presented on the next page. He is currently a lecturer in the Department of Theriogenology, University of Maiduguri, Nigeria. He is married with two adorable girls.

LIST OF PUBLICATIONS

Journal Articles

Published:

Innocent Damudu Peter, Abd Wahid Haron, Faez Firdaus Abdullah Jesse, Mokrish Ajat, Mark Hiew Wen Han, Wan Nor Fitri, Muhammad Sanusi Yahaya and Mohammed Saad M. Alamaary. Opportunities and challenges associated with faecal progesterone metabolite analysis. *Veterinary World*, 11(10): 1466-1472. doi: 10.14202/vetworld.2018.1466-1472.

Submitted:

Innocent Damudu Peter, Abd Wahid Haron, Faez Firdaus Abdullah Jesse, Mark Wen Han Hiew and Mokrish Ajat. Concentration of immunoreactive progesterone metabolite in feces of pregnant and non-pregnant cows. Submitted at Animal Reproduction Science.

Innocent Damudu Peter, Abd Wahid Haron, Faez Firdaus Abdullah Jesse, Mark Hiew Wen Han and Mokrish Ajat. Concentrations of immunoreactive progesterone metabolite in feces of pregnant and non-pregnant cows. Submitted at Pakistan Veterinary Journal.

Conference proceedings

Innocent Damudu Peter, Abd Wahid Haron, Faez Firdaus Jesse Abdullah, Mark Hiew Wen Han, Mokrish Moh'd Ajat. Comparison of faecal preservation and extraction methods employed in faecal progestagen analysis in cows. *Proceedings of the 30th Veterinary Association Malaysia Congress, 2018*. pp: 42

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