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ASSESSMENT OF OVARIAN FUNCTION IN THE SWAMP BUFFALO (BUBALUS BUBALIS)

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Assessment of ovarian function in the swamp buffalo (*Bubalus bubalis*)

by
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This thesis attached hereto, entitled "Assessment of Ovarian Function in the Swamp Buffalo (Bubalus bubalis)" prepared and submitted by Sharifuddin bin Abdul Wahab in partial fulfilment of the requirements for the degree of Master of Science, is hereby accepted.

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ABSTRACT

Since ovarian function in the water buffalo (Bubalus bubalis) has received considerable attention, a study was undertaken to: (a) relate ovarian contents to plasma progesterone levels during the oestrous cycle; (b) determine the accuracy of rectal palpation and the plasma progesterone assay for the assessment of ovarian function; and (c) establish the resumption of postpartum ovarian activity.

The experimental animals were drawn from a herd of swamp buffaloes, 5-8 years old which had calved between August 1981 and January 1982. For oestrus detection buffaloes were penned and tested daily between 20.00 hrs and 24.00 hrs using a vasectomized buffalo male fitted with a chinball marking device. Ovaries were palpated per rectum or examined by laparoscopy and progesterone levels in plasma were measured by a radioimmunoassay technique.

Sixteen cycling buffaloes were subjected to 32 ovarian examinations - laparoscopy and rectal palpation - as well as measurements of plasma progesterone levels. Gross changes in color, size and vasculature permitted the corpus luteum (CL) of the cycle to be graded into four age-dependent categories (CL I-IV) and the rise and fall in plasma progesterone levels reflected the development and regression of the CL.

The accuracy of diagnosing ovarian contents by rectal palpation and plasma progesterone levels was determined by examining the ovaries of 68 buffaloes. Thirty-eight (81%) of 47 rectal diagnoses of CL and 26 (90%) of 29 hormonal diagnoses (plasma progesterone levels >0.7 ng/ml of luteal tissue) were confirmed by laparoscopy. Seventeen (81%) of 21 rectal diagnoses of no CL and 25 (83%) of 30 hormonal diagnoses of no luteal tissue were also confirmed. The overall accuracy of

diagnosing ovarian function was 81 and 86 percent respectively for rectal palpation and the progesterone assay. Errors were mainly due to the rectal diagnosis of follicles as CL (19%) and the failure of CL to secrete luteal levels of progesterone.

Ovarian function in eleven suckled buffaloes were studied from parturition to 150 days postpartum. Plasma progesterone concentrations remained at basal levels during the first 30 days postpartum. An ovulatory cycle was observed in four buffaloes (36%) within 90 days postpartum, in five (45%) between 90 and 150 days postpartum whereas two (18%) animals were acyclic. The intervals from parturition to first ovulation and conception were 89 ± 53 and 112 ± 58 days respectively with an average of 1.4 matings to conceive. Buffaloes with corpora lutea diagnosed by rectal palpation and laparoscopy had luteal levels of plasma progesterone.

The results of this study showed that rectal palpation should continue as the preferred technique for the diagnosis of ovarian function in the buffalo under field conditions. Progesterone profiles are useful in diagnosing animals which are cycling but not detected in oestrus and animals which are anoestrous or are in early pregnancy. Laparoscopy is valuable for a detailed inspection of the ovaries and adnexa. The majority of suckled swamp buffaloes experience a period of postpartum anoestrus of 3 to 4 months duration prior to the resumption of ovarian cyclicity.

INTRODUCTION

Much of our knowledge on buffalo reproduction is based on studies in cattle. While this information has proved highly relevant to the buffalo, indiscriminate extrapolation of such findings to buffaloes must be viewed with extreme caution. The domestic water buffalo is an important animal in the agricultural economy of many Asian countries including Malaysia. Of the total population of 206,306 buffaloes in Malaysia, 3,048 are of river type kept mainly for dairy purposes while the remaining 203,258 are swamp buffaloes used primarily for draft power. Over 75% of buffaloe
Kelantan, Trengganu and Pahang where
harrowing and puddling of rice fields before planting. Buffalo meat production is of secondary importance though they contribute to about half the present beef supply of Malaysia.

Because of its ability to thrive under adverse conditions utilizing fodder of low nutritive value, its relatively good growth rate and better carcass weight than cattle, the Malaysian swamp buffalo has a good potential as a beef animal. However, as double cropping of rice, mechanization and illegal slaughter of breeding animals increase, the buffalo population in Malaysia is fast declining. Furthermore, poor reproductive rates and long calving intervals impose severe limitations on its exploitation for beef and milk production.

In cattle, a calving interval of approximately 365 days is essential for economic milk or beef production. A cow must be detected in oestrus by approximately 60 days postpartum and must be pregnant by 85 days if this goal is to be achieved. Similar calving to conception intervals could lead to optimal calving intervals in the swamp buffalo. This

necessitates a better understanding of ovarian function in the buffalo.

Studies on ovarian function in cattle has been based on oestrous behaviour, morphologic changes occuring in the ovaries detectable by rectal palpation, laparotomy or laparoscopy and by sensitive radioimmunoassay of ovarian and gonadotropic hormones in biological fluids, e.g. blood and milk. Of the ovarian steroids, progesterone concentration in blood plasma or milk has yielded the most information on ovarian function in cattle, because it reflects very closely the secretory activity of the corpus luteum.

Ovarian activity in the swamp buffalo has received considerable attention with the development of a method to induce oestrus and a radioimmunoassay for blood progesterone especially during the postpartum period. However, no studies have, so far, been made to relate progesterone levels and the presence or absence of luteal activity in the ovaries of the buffalo.

The objectives of this study are to correlate the ovarian contents observed by laparoscopy with rectal palpation of the ovaries and levels of plasma progesterone during the oestrous cycle; to assess the accuracy of rectal diagnosis of a corpus luteum or predicting luteal tissue by plasma progesterone levels; and to determine the patterns of postpartum ovarian activity in the suckled buffalo culminating in a resumption of cyclicity and conception.

REVIEW OF LITERATURE

Types of water buffaloes

The population of domesticated water buffaloes (Bubalus bubalis), in the world is at least 130 million. Most are nondescript animals that have not been selected or bred for productivity. There are two general types: the swamp (2n=48 chromosomes) and the river (2n=50 chromosomes) buffaloes.

Swamp buffaloes are slate grey, droopy necked and ox-like, with massive back swept horns. They are found from the Philippines to as far west as India. They wallow in water or mud puddle they can find or make. They are primarily employed as work animals, often used for pulling the plough and puddling rice fields. Swamp buffaloes are also used for meat but rarely for milk.

River buffaloes are found further west, from India to Egypt and Europe. Usually black or dark grey with coiled or drooping straight horns, they prefer to wallow in clean running water. They form the dairy type and produce more milk than swamp buffaloes. In rural areas of India and Pakistan, river buffaloes play an important role in rural economy as suppliers of milk and draft power. Buffalo butterfat is the major source of cooking oil in some Asian countries.

Contrary to many beliefs, both the types of buffaloes under farm conditions are very docile and can be frequently seen managed by small children driving them to rivers or ponds for cleaning in all Asian countries.

Reproductive performance of swamp buffaloes

In Malaysia, over 98 percent of the total population of 206,306

buffaloes are of swamp types (Jainudeen et al., 1977). Most of the buffaloes are concentrated in the rice growing states, where they are utilized for draft power in the rice fields (Fadzil, 1970).

The calving pattern of swamp buffalo in the rice growing state of Kedah showed that 75 percent of calvings occurred between the months of February and July (Fadzil, 1970) whereas at the Universiti Pertanian Malaysia buffalo farm, this seasonality was not observed (Jainudeen et al., 1977). The annual calf crop under village conditions was 43 percent (Fadzil, 1970). Under improved management conditions, the calving interval ranged from 393 to 700 days with an average of 532 days (Jainudeen, 1976b). Only very rarely will a swamp buffalo produce a calf each year. With an eleven month gestation length and a 6 to 7 month calving to conception interval, a suckled swamp buffalo produces on an average two calves every three years.

The long calving intervals, anoestrus and low reproductive rates limit a more efficient utilization of the buffalo in domestic agriculture and poses problems to the farmers (Fadzil, 1970; Jainudeen et al., 1977; Perera, 1981). Between day 60-150 postpartum three categories of ovarian activity were evident: corpus luteum (CL) with detected oestrus (21%); a CL with non-detected oestrus (42%); and absence of a CL with a non-detected oestrus or anoestrus (37%). A better understanding of the endocrinology of the postpartum period is needed to determine the contribution made by anoestrus to the long calving interval in the buffalo (Jainudeen et al., 1981).

Uterine involution and ovarian activity have been investigated in cattle by rectal palpation (Morrow et al., 1969; Wagner and Hansel, 1969) and plasma or milk progesterone profiles have been determined in post-partum dairy (Bulman and Lamming, 1978; Boyd and Munro, 1979) and beef (Redford et al., 1978; Rawlings et al., 1980) cows and in buffaloes by

rectal palpation, plasma progesterone (Perera et al., 1978; Kamonpatana et al., 1979; Jainudeen et al., 1981) and laparoscopy (Jainudeen et al., 1982a).

Reproductive endocrinology in the postpartum cow

In general, the endocrine regulation and morphological changes that occur during the early postpartum period in mammalian species show many similarities. Postpartum endocrinology has been studied in detail in cattle and since similar information is lacking for the buffaloes, it may be useful to briefly review the endocrine regulation of the resumption of ovarian activity in the postpartum cow.

In cattle, prostaglandin F_2alfa (PGF) is the key hormone released to terminate the luteal function during the late pregnancy. The trigger of PGF release in late pregnancy is probably due to a rise in oestrogen levels (Challis et al., 1972). The regression of the CL results in an abrupt decrease in the concentration of progesterone in the maternal peripheral blood plasma (Stabenfeldt et al.,1970; Edqvist et al., 1973). The PGF levels remain high and do not reach base-line levels until 10-20 days after delivery, however progesterone and oestrogen levels in maternal blood plasma decrease immediately following parturition (Edqvist et al., 1976; 1978).

The patterns of plasma luteinizing hormone (LH) and follicular stimulating hormone (FSH) in cows during the first 5 days postpartum show no clearly defined episodes of either LH or FSH irrespective of whether the cow is milked or suckled. Subsequently, the FSH level rises followed by an increased basal level of plasma LH. This may be related to a sporadic endogenous release of gonadotropic releasing hormone (GnRH) at frequent intervals giving rise to transient LH release with a return to basal levels of LH but with increased FSH release, causing a more sustained release in plasma FSH levels. Once

an increased level of FSH has occurred at an early stage in the post-partum milked cows, differences in plasma FSH are not considered as a factor limiting the onset of ovarian activity (Schams et al., 1978; Lamming et al., 1981).

The development of episodic release of LH may be a prerequisite for the onset of cyclic ovarian activity in the cow (Peters et al., 1981). The transition to ovarian activity may be due to an increased GnRH release leading to more frequent plasma LH episodes resulting in ovarian follicular activity and oestradiol secretion which enhances pituitary responsiveness to GnRH. In the postpartum milked cows, LH pulses typically appear in plasma at about day 10 but are delayed in intensively suckled cows which tend to undergo longer postpartum acyclic periods (Carruthers and Hafs, 1980; Peters et al., 1981). In the milked cow which exhibits ovarian activity by day 24 postpartum, there is a significant rise in basal levels of plasma LH prior to this time associated with an episodic LH release (Lamming et al., 1981). This is then followed either by a transient rise in milk progesterone (>3 ng/ml) or plasma progesterone (1-3 ng/ml) for up to 10 days for the normal luteal phase duration (Lamming, 1978; Webb et al., 1980; Lamming et al., 1981). This marks the luteal phase on the ovaries with a palpable CL per rectum. If a transient rise in progesterone occurred then this is followed by a similar preovulatory surge and ovulation (occassionally accompanied by oestrus) with a subsequent oestrous cycle of normal length.

Plasma concentrations of oestradiol-17 beta declined from prepartum peaks to basal levels by day 4-8 postpartum in suckled and milked cows (Arije et al., 1971; Smith et al., 1973). During the anovulatory phase of the postpartum period, large ovarian follicles are present (Kesler et al., 1979) associated with levels of oestradiol-17 beta as high as

those during oestrus (Rawlings et al., 1980). Although oestrogen levels are high, majority of otherwise normal cows do not show signs of behavioural oestrus or ovulation during this postpartum anovulatory period.

The regressing CL of pregnancy and plasma draining the ovaries contain very low levels of progesterone, 1-4 days postpartum (Labhsetwar et al., 1964). Thereafter, progesterone levels in peripheral blood remain at basal levels for a variable period (Arije et al., 1964; Robertson, 1972; Webb et al., 1980; Lamming et al., 1981) until the resumption of cyclic ovarian act In most cows, first ovulation is preceded by a short term elevation of progesterone (Pope et al., 1969; Donaldson et al., 1970; Lamming and Bulman, 1976; Webb et al., 1980). Once ovarian activity has been initiated, progesterone secretion by the corpus luteum exhibits a cyclic pattern with period of low progesterone (follicular phase) alternating with high progesterone (luteal phase).

Heat stress, suckling, season, nutrition and weaning are factors that govern the resumption of ovarian activity in most species of domestic animals. Thermal stress in cattle results in a variety of reproductive disturbances (Hafez, 1968; Jainudeen, 1976) such as a prolongation of the oestrous cycle (Madan and Johnson, 1973), a decrease in the intensity of oestrus or anoestrus under severe heat stress (Stott, 1962; Gangwar et al., 1965; Bond et al., 1972a). Heat stress prior to ovulation will block the ovulatory release of LH, thereby changing the temporal pattern of oestrus behaviour and ovulation (Baldwin and Sawyer, 1974).

An inhibitory influence of suckling upon the resumption of ovarian cycles in cattle has been reported by several investigators. The intensity of mammary stimulation is a major factor in prolongation of postpartum interval (Short et al., 1972; Wetteman et al., 1978). Post-

partum anoestrus is more common in suckled than machine milked cows, beef than dairy cows, cows milked frequently than milked twice daily (Carruthers and Hafs, 1980) and cows suckling twins than single calves (Wetteman et al., 1978). Calf removal hastens the resumption of ovarian cycles in beef cows (Smith et al., 1979; Carter et al., 1980) and in buffaloes (Jainudeen et al., 1982b). Milked buffaloes shows an earlier resumption of ovarian activity than suckled buffaloes (El-Fouly, 1976).

Reproductive endocrinology in postpartum buffalo

The endocrinological changes in the buffalo ovary during oestrus is similar to that of cattle. During the follicular phase in the buffalo, levels of plasma progesterone are low - <0.5 ng/ml. Following ovulation and as the CL starts to grow, there is a steady increase in the plasma levels of progesterone until it reaches a peak (≥2.0 ng/ml) and starts to fall to basal levels by the next oestrus (Perera, 1978; Kamonpatana et al., 1981; Jainudeen et al., 1982b; Jellinek and Avenell, 1982).

Oestrogen is the key hormone that induces the visual oestrous behaviour. Level of oestrogen was high during the follicular phase and fluctuates at low levels during the luteal phase (Kardjopranyoto et al., 1981).

Luteinizing hormone (LH) starts to fluctuate in an episodic manner from early follicular phase and an LH surge (20 ng/ml) was observed prior to ovulation. LH then falls to basal levels until the next follicular phase (Kaker et al., 1980; Kamonpatana et al., 1981; Razdan et al., 1981; Kardjopranyoto et al., 1981).

During pregnancy, the progesterone levels in buffalo plasma fluctuate between 0.8 ng/ml and 2.0 ng/ml. Oestrone levels start to increase steadily and reaches a peak at about 5 days antepartum (Kamonpatana et al., 1981; Perera, 1981). LH levels during pregnancy remained constantly low throughout pregnancy (Kamonpatana et al., 1981). Increased levels of PGF metabolites were recorded from 15 days antepartum

with a further increase during the last three days of pregnancy. The decline in levels of PGF metabolites occurs gradually and reaches basal levels 15-20 days postpartum (Perera, 1981). Progesterone and oestrone levels decline to basal levels on the day of parturition (Kamonpatana et al., 1981; Perera, 1981).

The CL of pregnancy (corpus albicans) regressed very rapidly following parturition. During the first month postpartum, the ovaries were less than 1 cm in length, smooth and were devoid of either follicles or CL. The plasma concentrations of progesterone were below 0.5 ng/ml. Between days 29 and 56 postpartum some animals (63%) possessed mature ovarian follicles, marked uterine tone and a discharge of cervical mucus without oestrus. However, ovarian structures palpated as CL were not associated with luteal phase progesterone levels (Jainudeen et al., 1982b).

During the postpartum period the plasma progesterone levels remained at basal values (<0.25 ng/ml) ranging from 115 to 210 days postpartum (Perera, 1981), up to 160 days postpartum (Kamonpatana et al., 1981) within 90 days postpartum (Jainudeen et al., 1982). During this period, the LH levels were below 2.05 ng/ml and mostly at the basal levels of 0.25 ng/ml whereas oestrone sulfate levels were around 0.01 ng/ml (Kamonpatana et al., 1981).

Assessment of ovarian function

The assessment of ovarian function can be studied by behavioural, clinical and endocrine techniques. A commonly employed method of studying ovarian activity in cattle is the observation of oestrus. This is also true for buffalo but overt signs of oestrus such as mounting other females or stand to be mounted by other females (homosexuality) is less common in buffaloes than cattle. Although matings do occur during daytime, the intensity of sexual activity is usually depressed during

the day. The modal length of oestrous cycle is approximately 21 days although cycles ranging from 17-24 days in length are not uncommon. Oestrus as determined by sexual receptivity to a male buffalo lasts 19 ± 2.1 hours with ovulation occuring 18.4 ± 1.4 hours after the end of oestrus. A feature of oestrus in the buffalo is the accumulation of an appreciable amount of clear mucus on the vaginal floor which unlike in cattle is not observed as a discharge from the vulva (Jainudeen, 1977; Jellinek and Avenell, 1982).

Several methods are available for the detection of oestrus in the cattle (Foote, 1975) but in the swamp buffalo, the method often used is a vasectomized bull (Jainudeen, 1977). The tail paint technique used in cattle (Mac Millan and Curnow, 1977) has also been applied to buffaloes (Jainudeen *et al.*, 1981).

The most frequently employed clinical technique for assessment of ovarian activity in cattle is rectal palpation. This technique in cattle has been adequately described in detail for the recognition of structures palpated (Zamjanis *et al.*, 1969). Murray (1959) presented a table of techniques and features to help differentiate between normal follicles, cysts and corpora lutea in the bovine ovary. The accuracy of rectal diagnosis of ovarian activity in cattle has been established by comparing with findings at slaughter (Belling Jr, 1964; Al-Dahash, 1977; Dawson, 1975). The accuracy of diagnosing CL *per rectum* is 89 percent based on slaughter (Dawson, 1975) and 70 percent of corpora lutea diagnosed by plasma progesterone were palpated (Boyd and Munro, 1979) and 85 percent accurate with milk progesterone levels (Watson and Munro, 1980).

Rectal palpation of the reproductive organs is extensively used in both the river and swamp buffaloes. In the buffalo, during oestrus (day 0), a follicle (approximately 10 mm in diameter) can be palpated *per rectum*, as a turgid area on the ovarian surface. Following ovulation

(days 4-6), a CL can be felt as a soft protrusion and with age, the CL is clearly demarcated from the rest of the ovary as a firm projection. At about day 17 following oestrus, the CL steadily decreases in size, becoming hard and nodular. The accuracy of rectal palpation in the diagnosis of ovarian contents in the swamp buffalo has not been established as the slaughter of buffalo cows is illegal in many Asian countries. Although plasma progesterone confirmed a diagnosis of anoestrus in buffaloes, no luteal levels of plasma progesterone were detected in buffaloes with ovarian structures palpated per rectum as CL's following first ovulation (Jainudeen et al., 1981).

Laparoscopy is the most recent technique available for the morphological study of ovarian activity in many domestic animals and is superior to laparotomy. The first report of laparoscopy in domestic animals was recorded in dogs in 1900's (Kellings, 1902) and in cattle (Megale et al., 1956). Laparoscopy is now an established technique for monitoring ovarian activity in cattle (Wishart and Snowball, 1973; Seeger, 1977), goats (Jarosz et al., 1971), sheep (Boyd and Ducker, 1973), horse (Witherspoon and Talbot, 1970), swine (Wildt et al., 1973) and recently in buffaloes (Jainudeen et al., 1982a).

The development of sensitive radioimmunoassay for the measurement of progesterone in biological fluids has enabled the study of ovarian activity in many species of domestic animals. A close relationship exist between ovarian activity and blood plasma progesterone levels in cattle (Boyd et al., 1979; Lamming and Bulman, 1976; Robertson, 1972) and in buffaloes (Perera, 1978; Kamonpatana et al., 1979; Jainudeen et al., 1981). The basis for the assay consists of the competition between a known amount of extracted hormone for binding sites on antibody molecules. The labelled hormone has a high proportion of ^{125}I or ^{3}H atoms in the molecule. After equilibration, the antibody-bound and unbound radioligand are separated and the radioactivity in the bound form can be

determined in a spectrometer and is inversely related to the endogenous hormone concentration.

Progesterone levels in blood plasma and milk have been used for the detection of postpartum acyclicity in cattle (Lamming, 1980), the diagnosis of problems associated with oestrus detection and timing of insemination (Appleyard and Cook, 1976; Foote et al., 1980), early identification of non-pregnant cows (Heap et al., 1973; Pope et al., 1976), buffaloes (Arora et al., 1980; Singh and Puthiyandy, 1980) and differential diagnosis of ovarian dysfunction (Booth, 1980).

During oestrus, plasma progesterone concentrations in cows were below 1 ng/ml (Pope et al., 1969) and in buffaloes less than 0.2 ng/ml (Perera et al., 1978; Kamonpatana et al., 1979; Alejandrino et al., 1981; Jainudeen et al., 1981). Progesterone levels in plasma in buffaloes follow a cyclic pattern similar to that of cattle (Edqvist et al., 1975) with the exception that the actual levels are much lower (Perera et al., 1978; Kamonpatana et al., 1979; Alejandrino et al., 1981; Jainudeen et al., 1981, 1982b).

MATERIALS AND METHODS

This study was conducted on suckled swamp buffaloes in the Universiti Pertanian herd at Pucong and calving between August 1981 and January 1982. The animals were 5 to 8 years old and weighed between 500 to 700 kg. They were maintained in paddocks of established pastures with facilities for wallowing and were fed a concentrate ration during the last month of gestation and lactation and all had access to trace mineral block which contained 10% phosphorus.

Oestrus was detected by visual observations, by a vasectomized buffalo bull fitted with a chin-ball mating device and by tail painting. The animals were left in the coral overnight with the vasectomized buffalo bull. A buffalo cow was said to be in oestrus (Day 0) if she stood to be mounted or marked by the bull or the tail paint rubbed off.

RECTAL EXAMINATION

The rectal examination of the ovaries was carried out as for cattle (Zemjanis, 1969). The ovaries were examined for morphological changes and measured for length (from pole to pole), width (from surface to surface) and height (from attached border to full border).

A follicle was diagnosed if it was smooth, round, slightly raised surface with fluctuation and was less than 10 mm in diameter. Follicles larger than 20 mm were considered as cysts.

The size and consistency of corpus luteum on rectal examination was recorded.

PLASMA PROGESTERONE ASSAY

Sample collection

A sample of jugular blood was collected into a 10 ml heparinized

evacuated tube (Termuro Corporation, Tokyo, Japan) after rectal examination of the ovaries. The blood was then centrifuged at 3,000 rpm for 15 minutes and the plasma separated, and stored at -20°C in a deep freezer pending analysis.

Chemicals and apparatus

Sodium azide was laboratory reagent grade from Sigma Chemical Co., St. Louis, USA and gelatin (granular powder, laboratory reagent) was from UCB, Brussels, Belgium. The charcoal suspension was prepared from Norit A charcoal and dextran T-70, both obtained from Sigma Chemical Co. Progesterone was purchased from Sigma Chemical Co., (1, 2, 6, 7-^{3}H) progesterone (82 Ci/mmol) from Amersham International Ltd., Amersham, UK. Scintillation fluid was prepared from toluene (May & Baker, England) and 2, 5-diphenyloxazole (PPO) Sigma Chemical Co., USA. Petroleum ether (AnalaR) with a boiling point from 40 to 60°C was purchased from BDH Chemicals Ltd., Poole, England.

Pipetting was carried out with "Pipetman" (Gilson, France) or "Quickpette" (Helen Lab., Texas, USA).

Glass tubes were used for extraction (12 x 100 mm) and the assay (10 x 75 mm). For counting 20 ml glass scintillation vials were used.

The equipment used included a supermixer (Lab Line Instruments, Inc., Melrose Park, Illinois, USA), multiple vortexer (Scientific Manufacturing Industries, USA, model 2601), a refrigerated centrifuge (International Equipment Co., USA), a liquid scintillation B-counter (Packard Instruments, USA, model C-300) and a programmable calculator (T-59, Texas Instruments, USA).

Preparation of reagents

Phosphate-buffered saline (PBS) containing 0.1 M sodium phosphate (pH 7.0), with 0.9% NaCl and 0.1% sodium azide (Abraham et al., 1977)

was prepared as follows:-

To a 2-litre volumetric flask was added 32.7 g sodium phosphate dibasic heptahydrate (MW 268), 10.8 g sodium phosphate monobasic monohydrate (MW 138), 2.0 g sodium azide (MW 65) and 18.0 g sodium chloride (MW 58). Deionized water was added up to a total volume of 2 litres. The assay buffer also contained 0.1% gelatin (PBSG). The buffer was stored at $4^{o}C$.

Radioactive and non-radioactive progesterone were diluted with ethanol and stored in a freezer ($-20^{o}C$). When labelled progesterone was used as tracer in the RIA, it was evaporated to dryness in a flask and PBSG was added to yield approximately 20,000 dpm ^{3}H-labelled progesterone in 100 µl of assay buffer.

To prepare dextran-coated charcoal suspension, 625 mg Norit A and 62.5 mg dextran T-70 were added to 100 ml of assay buffer, mixed in a magnetic stirrer and stored at $4^{o}C$ in a refrigerator until used. When in use the suspension was kept on a stirrer in order to obtain a homogenous charcoal-buffer solution.

The scintillation fluid contained 4 g PPO per litre of toluene.

Quality control (QC) samples were two pools of buffalo plasma containing >2.0 ng/ml (high) and $\geq$1.0 ng/ml (low) concentration of progesterone. Aliquots from each pool were stored at $-20^{o}C$ in small amounts sufficient for each assay.

Antisera

The antisera for the assay was donated by Dr. L.E. Edqvist, University of Agricultural Sciences, Uppsala, Sweden. It was prepared in a ewe immunized against 11-hydroxyprogesterone hemisuccinate-bovine serum albumin. The cross-reactivity with progesterone was 100%; 17α -hydroxyprogesterone, 9.5%; 11α -hydroxyprogesterone, 3.5%; 11 -desoxycorticosterone, 3.1%; 20α -hydroxyprogesterone, 1.3%; and other steroids

less than 0.1% (see Castellanos & Edqvist, 1978).

Assay procedure

Plasma levels of progesterone were measured by a RIA procedure as described by Abraham (1977) and Castellanos and Edqvist (1978) and adapted to the buffalo (Jainudeen et al., 1981). Duplicate samples of plasma (500 µl) were extracted with 2 ml of petroleum ether by intensive shaking in a Vortex. After the plasma was frozen at -20°C in a freezer, the s tubes and the petroleum ether was evaporated to dryness in a 37°C water bath under a stream of filtered air. Next, 500 µl of PBSG and 100 µl of antiserum (1:3000) were added to all assay tubes. The contents of the tubes were briefly mixed and incubated at room temperature for 15 min. before adding 100 µl of (^{3}H)-progesterone (20,000 dpm). After incubation of samples overnight at 4°C in a refrigerator, 200 µl of dextran-coated charcoal suspension was added to each tube at 4°C, briefly mixed on Vortex, and immediately centrifuged for 10 min. at 800 x g in a refrigerated centrifuge (4°C). The supernatant containing protein-bound progesterone was decanted into 20 ml scintillation glass vials, and radioactivity counted for 10 min. after the addition of 5 ml of toluene-PPO in a liquid scintillation spectrometer. Duplicate samples from the two quality control pools were similarly treated.

A standard curve was assayed simultaneously with each set of unknown samples. From stock solutions of progesterone in ethanol (100 pg/ml and 1000 pg/ml) aliquots were pipetted into duplicate tubes to obtain 25, 50, 100, 150, 200, 300, 400, 500, 600, 800 and 1000 pg of progesterone per assay tube after evaporating to dryness with 2 ml of petroleum ether. The tubes were then assayed as for unknown samples.

Tubes used for determining non-specific binding (NSB) contained assay buffer (600 µl) and 100 µl of tracer. The zero count (B_o) tubes