Estimation of progesterone in buffalo milk and its application to pregnancy diagnosis

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Summary. Milk samples were collected on Days 20, 24, 28 and 40 after insemination. The percentage of fat and the progesterone concentration in the milk were correlated ($P < 0.01$). Progesterone concentration in the milk of pregnant buffaloes (24.83 ± 3.85 ng/ml) was significantly higher than that in non-pregnant animals (2.89 ± 1.25 ng/ml) on Day 20 and the difference between the two increased with time after insemination. The detection of non-pregnant animals was 100% successful at all times but the diagnosis was correct for 66, 68, 81 and 83% of animals tested on Days 20, 24, 28 and 40 respectively and predicted as pregnant.

Introduction

Since the report that progesterone can be measured in cow milk (Heap & Linzell, 1966; Heap, Linzell & Slotin, 1969; Heap, Bedford & Linzell, 1975), there has been much research to evolve a precise and quick method for the estimation of progesterone in milk and to correlate the values with the reproductive state of the cow. Laing & Heap (1971) first reported that progesterone concentration in milk might provide a method for early pregnancy diagnosis. Heap, Gwyn, Laing & Walters (1973) proposed the use of radioimmunoassay of progesterone without ether extraction as a rapid method of measuring progesterone in milk and many workers have now established that the progesterone concentration in the milk of cows can be used to detect animals in pregnancy (Thibier, 1974; Schiavo, Matuszczak, Oltenacu & Foote, 1975; Heap, Holdsworth, Gadsby, Laing & Walters, 1976; Pennington, Spahr & Lodge, 1976; Pope, Majzlik, Ball & Leaver, 1976; Lamming & Bulman, 1976; Laing, 1976; Dobson & Fitzpatrick, 1976). Almost all these workers have suggested that accurate diagnosis of pregnancy is possible at 21–24 days after mating.

There appears to be no report available on the progesterone concentration in the milk of buffaloes and the aim of the present study was to measure progesterone concentrations in buffalo milk and correlate it with the state of pregnancy.

Materials and Methods

Samples

Murrah buffaloes (*Bubalus bubalis*) were chosen for this study from the herd maintained at this Institute. Such buffaloes have an oestrous cycle of about 21 days.

Samples of first milk and full milk were initially collected at the morning (05:00 h) and evening (18:30 h) milking on Days 20, 24 and 28 after artificial insemination. The animals were inseminated, with 1 ml semen containing $50\text{--}100 \times 10^6$ spermatozoa, 12–18 h after the start of oestrus. Subsequent samples of full milk were collected at the evening milking only on Days 20, 24, 28 and 40 and stored in a deep freeze.

Assay

The antiserum to progesterone was prepared in rabbits against progesterone-11 β -succinyl-bovine serum albumin and was obtained as a freeze-dried powder from New England Nuclear (Boston, Massachusetts). The cross-reactions of the antiserum with other hormones (progesterone 100%) were reported to be as follows: 17-hydroxyprogesterone, 4.3%; 16 α -hydroxyprogesterone, 1.3%; 20 α -dihydroprogesterone, 0.6%; pregnenolone, 11-deoxycortisol and testosterone, 0.1%; and cortisol <0.1%.

Another antiserum, subsequently obtained from Dr B. V. Clifton (I.C.I. Cheshire, England), was raised in a goat immunized against progesterone 11 α -succinyl-bovine serum albumin. The cross-reactions with other hormones (progesterone 100%) were as follows: 11 α -hydroxypregn-4-en-3,20-dione 177%; 11 β -hydroxypregn-4-en-3,20-dione, 35%; deoxycorticosterone, 12.5%; corticosterone, 3.8%; 5 β -pregnane-3,20-dione, 2.5%; oestradiol-17 β , 0.7%; 16 α -hydroxyprogesterone, 0.5%; 20 β -hydroxypregn-4-en-3 one, 0.2% and other steroids <0.1%.

The assay for progesterone was essentially as described by Heap *et al.* (1976). The collected milk samples were thawed to room temperature and 20 μ l were pipetted into a 7 ml glass test-tube and diluted with 500 μ l of 0.1 M-phosphate buffer (3.04 g NaH₂PO₄ · 2H₂O, 5.43 g Na₂HPO₄ · 2H₂O, 4.5 g NaCl, 0.5 g NaN₃ and 0.5 g gelatin in 500 ml distilled water) adjusted to pH 7.0 with 1 N-NaOH.

The progesterone antiserum was made up in 100 μ l 0.1 M-phosphate buffer and added. The dilution of antiserum used was sufficient to bind 30–40% of 20 000 d.p.m. [1,2,6,7-³H]progesterone (sp. act. 97.9 Ci/mmol; New England Nuclear). The tubes were incubated at 16–18°C for 30 min. Then another 100 μ l [1,2,6,7-³H]progesterone (20 000 d.p.m.) in phosphate buffer were added and vortexed. The samples were then incubated at 4°C for 14–18 h. The reaction was terminated by placing the tubes in crushed ice. After incubation, 1 ml charcoal suspension containing 250 mg Norit A charcoal (ICN Pharmaceuticals Inc., Cleveland, Ohio) and 25 mg dextran T-70/100 ml 0.1 M phosphate buffer was added and the mixture centrifuged at 4°C. Then 0.5 ml supernatant was transferred to counting vials and 10 ml toluene-Triton X-100 (2:1 v/v) scintillation fluid were added. The radioactivity was measured in a Scintillation spectrometer (Model 3330, Packard Tri-Carb) for 1 min.

For the standard curve, progesterone (Sigma Chemical Co., St. Louis, U.S.A.) was dissolved in distilled alcohol and a small measured fraction was dried under nitrogen, dissolved in phosphate buffer and serially diluted to provide 0, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0 and 4.0 ng samples in 0.5 ml buffer. These samples were mixed in 20 μ l milk collected from a non-pregnant non-cyclic buffalo, the milk of which contains little or no progesterone, and treated in the same way as samples being tested. The corresponding binding percentages were 37.1, 27.0, 26.1, 18.6, 17.2, 15.0, 12.1 and 9.8. The progesterone values for the unknown samples were obtained by interpolation from the standard curve. A zero standard without any progesterone and a blank excluding antiserum were also included in each assay run. The sensitivity of the assay was 0.5 ng/ml.

Estimation of fat

Milk fat was estimated by the Gerber method described by the Indian Standards Institution (1958).

Results

Progesterone in first and full milk samples

The results for pregnant and non-pregnant buffaloes are shown in Table 1. In all the pregnant animals, milk progesterone concentrations and fat content were significantly higher in the full-milk than in the first-milk samples (Table 2). Evening values were consistently higher

than those for morning samples and the difference was significant (Table 2). There was a significant ($P < 0.01$) correlation between milk progesterone concentration and fat content for all samples except that for evening full milk on Day 28. In the non-pregnant animals the full-milk fat values were significantly higher than those for first-milk samples (Table 2), but the differences for the progesterone concentrations were not as great as for pregnant buffalo cows. There was a significant correlation ($P < 0.01$) between milk fat and progesterone level for all samples except that for morning full milk on Day 28.

Table 1. Mean $\pm$ s.e.m. progesterone concentration (ng/ml) and fat in first and full milk taken at the morning and evening milkings from 17 pregnant and 14 non-pregnant buffaloes at different times after insemination

	Time of day (h)	Day 20		Day 24		Day 28	
		First milk	Full milk	First milk	Full milk	First milk	Full milk
Progesterone (ng/ml)							
Pregnant	05:00	9.5 ± 2.5	21.0 ± 3.4*	11.3 ± 2.9	24.1 ± 2.8*	19.0 ± 2.9	29.4 ± 3.9*
	18:30	11.6 ± 2.1	25.2 ± 2.8*	12.4 ± 2.0	26.1 ± 3.8*	21.6 ± 3.2	33.2 ± 4.2*
Non-pregnant	05:00	0.9 ± 0.3	1.5 ± 0.9	0.98 ± 0.9	1.7 ± 1.1	1.0 ± 0.4	1.7 ± 1.1
	18:30	1.1 ± 0.6	2.1 ± 1.3	1.2 ± 0.8	2.3 ± 0.98*	1.3 ± 0.98*	2.7 ± 1.4
Fat (%)							
Pregnant	05:00	4.5 ± 0.2	8.5 ± 0.3*	4.5 ± 0.1	8.6 ± 0.3*	4.3 ± 0.2	8.4 ± 0.2*
	18:30	4.7 ± 0.2	8.6 ± 0.3*	4.5 ± 0.1	8.6 ± 0.3*	4.6 ± 0.1	8.6 ± 0.2*
Non-pregnant	05:00	4.2 ± 0.2	8.4 ± 0.4*	4.9 ± 0.2	8.7 ± 0.3*	4.2 ± 0.2	8.7 ± 0.4*
	18:30	4.2 ± 0.2	8.5 ± 0.4*	4.3 ± 0.2	8.6 ± 0.3*	4.4 ± 0.1	8.7 ± 0.3*

* Significantly different from corresponding first milk sample $P < 0.01$.

Table 2. Analysis of variance of fat and progesterone values of pregnant and non pregnant buffaloes

Source of variation	d.f.	Pregnant buffaloes				Non-pregnant buffaloes			
		Progesterone concentration		Fat %		Progesterone concentration		Fat %	
		M.S.S.	F value	M.S.S.	F value	M.S.S.	F value	M.S.S.	F value
Among replicates	16	7.98	2.45**	1.96	3.29**	13	0.30	1.77	8.92
Among times	1	207.03	63.62**	3.34	5.60*	1	10.32	60.33**	0.23
Among periods	2	1224.93	376.43**	0.31	5.14*	2	1.27	7.41**	0.54
Among types of milk	1	7316.30	2248.39**	849.54	1423.94**	1	34.92	203.20**	780.02
Interactions									
Timings $\times$ periods	2	17.00	5.23*	0.23	0.38	2	0.29	1.67	0.03
Timings $\times$ type of milk	1	7.74	2.38	0.26	0.44	1	2.95	17.18**	0.20
Periods $\times$ type of milk	2	48.07	14.77**	0.03	0.06	2	0.18	1.07	0.12
Timings $\times$ periods $\times$ type of milk	2	7.51	2.31	0.001	0.016	2	0.07	4.20*	0.002
Error	176	3.25	—	0.59	—	143	0.17	—	0.18

* $P < 0.05$.

** $P < 0.01$.

Progesterone levels in pregnant and non-pregnant animals

The results in Table 3 indicate that there was a clear difference in the milk progesterone concentrations in pregnant and non-pregnant animals. Concentrations of progesterone rose

steadily in the pregnant animals with the advance of pregnancy, while those in the non-pregnant animals decreased.

Pregnancy test

The animals selected for this study were classified as pregnant or non-pregnant on the basis of their milk progesterone levels. Those with < 7 ng progesterone/ml were considered to be non-pregnant. The results are shown in Table 4. Animals diagnosed as non-pregnant were checked by physical examination and whether they returned to oestrus. The test was completely efficient for detection of non-pregnant animals, but was less successful for pregnant buffaloes, being 65–83% correct. The accuracy at Day 28 was nearly as good as that at Day 40.

Table 3. Concentrations (mean $\pm$ s.e.m.) of progesterone in milk (full-milk evening) samples (no. in parentheses) of pregnant and non-pregnant buffaloes at various times after insemination

Day after insemination	Progesterone (ng/ml)	
	Pregnant	Non-pregnant
20	24.8 $\pm$ 3.9 (21)	2.9 $\pm$ 1.3 (24)
24	27.5 $\pm$ 3.4 (19)	2.5 $\pm$ 1.1 (23)
28	28.5 $\pm$ 2.7 (22)	2.4 $\pm$ 1.0 (26)
40	32.4 $\pm$ 4.2 (20)	1.8 $\pm$ 0.6 (28)

Table 4. Diagnosis of pregnancy in buffalo cows from progesterone concentrations in milk samples

Day after insemination	No. of cows tested	Predicted pregnant*		Predicted non-pregnant†	
		Total	Correct (%)	Total	Correct (%)
20	56	32	21 (65.6)	24	24 (100)
24	51	28	19 (67.9)	23	23 (100)
28	53	27	22 (81.5)	26	26 (100)
40	52	24	20 (83.3)	28	28 (100)

* Milk progesterone concentration of > 7 ng/ml.

† Milk progesterone concentration of < 7 ng/ml.

Discussion

It is clear that most of the progesterone in buffalo milk is associated with the milk fat. Heap & Laing (1974) reported that the progesterone concentration of cow milk could be related to fat content of the milk in that 75% of the hormone was present in the fat and 25% in casein. Our results for buffalo are in good agreement with the values reported by Heap *et al.* (1976) and Pennington *et al.* (1976) for cows.

The progesterone concentrations in buffalo milk are in agreement with those of Hoffman, Gunzler, Hamburger & Schmidt (1976) for cow milk, although Heap *et al.* (1976) suggested that the values obtained for progesterone in milk may be over-estimates because of the presence of metabolites of progesterone which might cross-react with the antiserum. The difference in the milk progesterone levels in gravid and non-gravid animals was reported earlier by several workers (Dobson & Fitzpatrick, 1976; Gunzler & Hamburger, 1976; Lamming & Bulman, 1976).

Successful diagnosis of non-pregnant buffaloes was possible by Day 20 after insemination, and the success rate of pregnancy diagnosis is similar to the values obtained for cows (Heap *et al.*, 1976; Hoffman *et al.*, 1976; Pennington *et al.*, 1976; Koefoed-Johnsen, 1976). The error arising from false positive observations at Day 20 and Day 24 after insemination was substantial. The occurrence of false positive predictions, related to high (> 7 ng progesterone/ml) milk progesterone concentrations even at 4 weeks after insemination, is probably due to embryonic loss as reported for lactating cows (Pope & Hodgson-Jones, 1975; Heap *et al.*, 1976).

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