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Progesterone Concentration in Milk and Blood During Pregnancy in Cows[1] (38103)

O. J. GINTHER, L. NUTI, B. C. WENTWORTH, AND W. J. TYLER

Departments of Veterinary Science, Poultry Science, and Dairy Science, University of Wisconsin, Madison, Wisconsin 53706

The concentration of progesterone in the milk of various species apparently has received only limited study (1, 2). In the present project progesterone was assayed in the milk and blood plasma of cows during pregnancy.

Materials and Methods. Blood and milk samples were collected from 56 Holstein–Friesian cows during their second or third pregnancy. Samples were collected from eight cows on each of the following days of pregnancy: 30, 60, 90, 120, 150, 180, and 210. Collections were made during the winter (November and December) and the cows were fed stored roughages (silage, hay) and grain consisting of 16% crude protein. The cows were not given any exogenous hormones or other drugs for several months prior to collection of samples.

Milk (100 ml) and blood (20 ml) were collected approximately 2 hr after the morning milking. The blood was heparinized and both samples (milk and blood) were put immediately into an ice water bath and were stored in a refrigerator. The samples were transported to the laboratory every 3 or 4 days. The plasma was removed from the blood by centrifugation and the milk and plasma samples were stored in a freezer.

Concentration of progesterone in the milk and plasma samples was measured by radioimmunoassay using a modification described by Abraham (3). Radioactive progesterone (1,2-^{3}H-progesterone, New England Nuclear) was purified on microcolumns of Sephadex LH-20 (Pharmacia). Approximately 2000 cpm of tritiated progesterone in 0.10 ml of a solution of 0.1% gelatin in phosphate buffer (0.1% PBSG) were added to 4 ml of milk and 3 ml of plasma as an internal standard for recovery estimates. The milk samples were extracted by vortex mixing for 30 sec with 8–10 ml of diethyl ether followed by centrifugation at 2000 rpm for 10 min. The ether phase was removed with a disposable Pasteur pipet and evaporated to dryness under a stream of air in a water bath (38 C). This extraction procedure was repeated two more times. Plasma samples were extracted like the milk samples except that 5 ml of diethyl ether was used each time.

Microcolumns of Sephadex LH-20 were prepared in 3-ml syringes which were plugged with glass fiber filters (grade 934 AH, Reeve Angel). Extracts from the plasma and milk samples were chromatographed on microcolumns containing 0.9 g of Sephadex LH-20 eluted with methylene dichloride: methanol (98:2). The progesterone fractions (1–8 ml) were collected from the columns and were dried and rechromatographed.

The Sephadex LH-20 (0.9 g) which was used for column chromatography of the progestins was slurried with 4–5 ml benzene: methanol (85:15). The columns were washed with 8–10 ml eluting solvent (hexane:benzene:methanol, 85:5:4) until all the air bubbles were removed. Another glass fiber filter was placed on top of the Sephadex and the columns were washed with an additional 10 ml of eluting solvent. The sample was applied to the column using two successive additions of 0.5 ml and the appropriate progesterone fractions (4–7 ml) were collected in scintilla-

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tion vials. The column effluent was evaporated to dryness and 2.0 ml of 0.1% PBSG was added and allowed to equilibrate for a minimum of 24 hr. After incubation, duplicate 0.1- and 0.2-ml aliquots of each fraction were dispensed into assay test tubes for quantitation. At the same time, 1.0-ml aliquots were removed and used to determine a recovery estimate which was used to correct for procedural losses.

An antiserum to the antigen (progesterone-11α-BSA conjugate; HI Laboratories) was prepared in sheep. The antibody dilution which was selected (1:2,000) bound 20–30% of 10,000 cpm of labeled steroid in the tubes containing no radioinert steroid. Two-tenths milliliter of diluted antiserum was added to each of the standard tubes and the sample tubes which were then gently vortexed. The tubes were incubated for 1 hr at 4 C. The addition of the appropriate trace solution containing approximately 10,000 cpm in 0.1 ml of 0.1% PBSG was followed by gentle agitation and the tubes were incubated at 4 C for a minimum of 6 hr. After incubation, 0.6 ml of dextran-coated charcoal solution (4) was added and the samples were mixed vigorously for 5 sec. This was incubated at 4 C for 60 min and was then centrifuged at 3000 rpm for 10 min at 4 C. One milliliter of supernatant was transferred to a counting vial and 5–7 ml of scintillation fluid was added. The vial was vortexed and then counts were made after a minimum equilibration period of 6 hr.

The samples were run in eight assays. Each assay consisted of the milk and blood samples from one cow on each of the 7 days of pregnancy. A reagent blank and an aliquot from a pool of bovine plasma (interassay standard) was included in each assay. The intraassay coefficient of variation was determined by assaying an aliquot from the pool of plasma five times.

Validity of the progesterone assay was tested as described by Bliss (5) and modified by Staigmiller (6). Two replicated standard curves were made using two tubes at five doses ranging from 0.125 to 2.000 ng. A logit transformation of percentage bound was used as the ordinate and log dose as the abscissa. Sums of squares were computed for dose, replicate, and interaction of replicate and dose. The dose sum of squares was partitioned into a linear and a nonlinear component. An F test (5) was used to determine whether there was significant deviation from linearity. Milk and blood plasma samples (unknowns) were taken from one cow at random from each of the following stages: days 4, 10, and 16 of diestrus and days 30, 120, and 210 of pregnancy. The 12 unknowns (six milk and six plasma) were assayed at two doses and heterogeneity of regression among unknowns was checked by analysis of variance. The interaction of sample with dose was used as a measure of the variance caused by differences in dose response among the unknown samples. The mean dose response for the unknowns was compared to that of the standard curves for parallelism using the Student t distribution (7). The standard curve was used to convert the response of the unknowns into hormone potency.

Results. The reagent blanks were close to zero and did not give a significant dose response. The coefficient of variation for the interassay standard and intraassay standard was 33.7% and 11.7%, respectively.

The standard curves for the validity test were linear ($F = 0.02$ whereas an $F = 9.28$ was required for significant deviation from linearity) and the two curves were, therefore, combined. The interaction of unknown samples with dose was not significant indicating that the response among the unknowns was not heterogeneous ($F = 1.84$ whereas an $F = 2.69$ was required for significance). The t value (0.50) from the comparison of the mean dose response of the unknowns with the standard curve was not significant indicating that the regression for the unknowns and the regression for the standard were not heterogeneous.

Progesterone was higher ($P < 0.005$) in milk (21.2 ng/ml) than in blood plasma (5.3 ng/ml) averaged over all days of pregnancy (Table I). There were no significant differences among the days of pregnancy averaged over the two fluids (milk and blood plasma). The interaction between day of pregnancy and type of fluid (milk vs plasma) tended to be significant ($P < 0.1$). This tendency seemed to be due primarily to lower levels of progesterone in milk on days 150, 180, and 210 than on days 30, 60, 90, and 120 with the level in plasma remaining constant over all days. The correlation between progesterone levels in

TABLE I. Concentration of Progesterone in Milk and Blood Plasma of Cows During Pregnancy.[a]

Day of pregnancy	Progesterone in milk (ng/ml)		Progesterone in blood plasma (ng/ml)	
	Mean	SE	Mean	SE
30	25.1	± 3.5	5.1	± 0.7
60	23.0	± 1.8	5.3	± 0.4
90	26.2	± 4.9	5.3	± 0.5
120	22.9	± 2.4	5.0	± 0.9
150	18.0	± 2.7	5.6	± 0.4
180	15.1	± 1.8	5.0	± 0.6
210	18.4	± 1.9	5.7	± 0.6

[a] Milk and plasma assayed in eight cows at each day of pregnancy. Differences among days of pregnancy averaged over milk and plasma concentrations (not significant). Difference between milk and plasma averaged over all days of pregnancy ($P < 0.005$). Interaction of day of pregnancy and milk vs plasma ($P < 0.1$).

milk and plasma within cows was not significant ($r = +0.07$).

Discussion. On most dairy farms milking is discontinued 6–8 wk prior to parturition. The last day of sampling during pregnancy (day 210) in the present project, therefore, was near the time when milking would be discontinued. On the average, the concentration of progesterone in pregnant cows was four times higher in milk than in blood plasma. The concentration of progesterone in the blood (mean, 5.3 ng/ml) is in agreement with what has been reported for pregnant cattle by some authors (8, 9), but appears to be several nanograms lower than what has been reported by others (10, 11). Laing and Heap (12) have reported on the concentration of progesterone in the milk of pregnant cattle based on a competitive protein binding assay. Blood samples were not included. The means for milk reported herein for the seven stages of pregnancy (15.1–26.2 ng/ml for days 30–210) fall within the range reported by Laing and Heap (7.1–35.6 ng/ml for days 35–215).

It is not known whether the high level of progesterone in milk is due to concentrating mechanisms for transfer of progesterone from blood to milk or to synthesis of progesterone by the mammary gland. Mammary tissue apparently is able to remove progesterone from the blood. In goats, about 20% of the progesterone which is produced by the ovaries is removed from the blood by the mammary glands (13). The mammary glands of rabbits have been reported to remove labeled progesterone from the blood (14). The concentration of the labeled progesterone was much higher in the mammary veins than in the vena cava indicating a longer half-life of progesterone in mammary tissue than in the general circulation. In women, malignant, but not normal mammary tissue, formed progesterone from cholesterol *in vitro* (15). Furthermore, it has been reported that the mammary glands of two goats were able to synthesize progesterone from pregnenolone (16). Radioactive pregnenolone was infused into the artery of the transplanted mammary glands and small amounts of the radioactive progesterone were detected in the venous outflow. The above reports, however, did not consider whether progesterone which was removed from the blood or possibly produced by the mammary glands appeared in the milk. Williams (17) reported that extremely small quantities of progesterone or its metabolites were found in the milk of two cows after labeled progesterone was injected into the jugular vein, and concluded that milk was of no importance in the excretion of progesterone or its metabolites. Labeled progesterone infused into the arterial system of the mammary glands of goats reportedly appears in milk, but in low concentrations (18). Similarly, only negligible amounts of radioactivity were found in milk of ewes after treatment with a labeled progestin, whereas high radioactivity was found in feces and urine (19).

In the present project, the level of progesterone in milk was higher than in plasma for each of the 56 cows. This finding is compatible with the hypotheses that mammary tissue removes progesterone from the blood and excretes it into the milk or mammary tissue synthesizes progesterone and secretes it into the milk. It is noteworthy that the magnitude of the difference in progesterone concentration between milk and plasma varied considerably among cows. The difference in progesterone concentration between milk and plasma within cows was quite variable and ranged from 1.6 to 44.3 ng/ml. The correlation between levels in blood and milk was not significant. It should be noted, however, that milk, as contrasted to blood, is pooled over a period of time. In the present project, the milk was from a pool which was secreted for approximately 2 hr and in addition probably contained residual milk from the previous milking. Approximately 20% of the milk remains as residual milk after a normal, thorough milking (20).

It seems likely that the assay measured progesterone with minimal cross-reaction with other steroids. The antigen used for immunization and as a standard was a pure, synthetic progesterone. The cross-reaction of 18 gonadal and adrenal steroids with the antiserum which was used herein has been studied by Staigmiller (6). Four progestins had a cross-reaction greater than 1%: 5β-pregnane-3,20-dione (20.0%), pregnenolone (10.0%), 17α-OH progesterone (4.8%), and 20β-OH-pregn-4-ene-3-one (2.4%). The Sephadex columns which were used in the present project removed at least the latter three progestins. Although the validity test indicated parallelism among unknowns, whether milk or plasma, and between the unknowns and the standard the possibility of cross-reactions cannot be completely eliminated. Therefore, and in view of the high values obtained from milk, confirmatory study involving determination of steroid profiles in milk by gas–liquid chromatography is indicated.

Summary. The concentration of progesterone in milk and blood plasma was determined by radioimmunoassay in 56 pregnant cows. Samples were from eight cows on each of the following days of pregnancy: 30, 60, 90, 120, 150, 180, and 210. Validity of the assay was indicated by parallelism among unknowns, whether milk or plasma, and between unknowns and the standard. Progesterone was higher ($P < 0.005$) in milk (21.2 ng/ml) than in blood plasma (5.3 ng/ml) averaged over all days of pregnancy. The difference among days and the interaction of day and type of fluid (milk vs plasma) were not significant.

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