Concentrations of oestrone sulphate in milk during pregnancy in dairy cows

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ABSTRACT

A monoclonal antibody to oestrone sulphate (ES) was generated, and used in the development of a radioimmunoassay to measure ES concentrations in whole milk of cows. The mean ± s.e.m. concentration of ES in milk samples from non-pregnant cows was 58 ± 5 pg/ml (n = 20). In pregnant cows, ES concentrations in milk samples rose progressively from a mean ± s.e.m. value of 99 ± 10 pg/ml (n=20) at 40 to 60 days of pregnancy, to a plateau value of approximately 1000 pg/ml at about day 160 of pregnancy. Examination of the ranges of ES concentrations in milk sampled from non-pregnant and pregnant cows indicated that all non-pregnant cows and 46% of cows <120 days pregnant had milk ES concentrations <125 pg/ml. However, only 4% of cows ≥ 120 days pregnant had milk ES concentrations <125 pg/ml. Measurement of ES concentrations in milk samples taken at least 120 days after mating/insemination could provide an accurate indication of pregnancy status in New Zealand dairy cows.

Keywords Oestrone sulphate, monoclonal antibody, cows, milk, pregnancy, radioimmunoassay.

INTRODUCTION

The steroid metabolite oestrone sulphate (ES) is produced by the foetal-placental unit in increasing amounts as pregnancy progresses in several species including cows, sheep, goats and pigs (Sasser & Ruder, 1987). The ES enters the maternal circulation and can be readily measured in biological fluids such as blood and milk sampled from the mother. Overseas studies of pregnant dairy cows have shown that ES concentrations in milk increase above non-pregnancy levels approximately 100 days after mating/insemination, and remain high for the remainder of gestation (Holdsworth *et al.*, 1982; McCaughey *et al.*, 1982). Thus, measurement of ES concentrations in milk can be a useful means of identifying the pregnancy status of cows. At present, no data is available on the ES concentrations in milk throughout pregnancy in New Zealand dairy cows which are fed a sole diet of pasture, and produce milk with a high solids content. The purpose of this study was to a) develop a radioimmunoassay for ES in whole milk using a monoclonal antibody, and b) measure ES concentrations in milk samples obtained from non-pregnant and pregnant dairy cows.

MATERIALS AND METHODS

Generation of monoclonal antibody to oestrone sulphate

A monoclonal antibody to ES was generated using standard methodology (Campbell, 1984). Briefly, Balb/c mice were actively immunized with an ES-protein conjugate antigen in Freund's complete adjuvant. Binding of tritiated (^{3}H) ES, in conjunction with dextran-coated charcoal separation of bound and free ^{3}HES, was used to monitor ES antibody titres in plasma samples taken from the mice at fortnightly intervals. Three days after a final booster of antigen, given intravenously in phosphate buffered saline, splenocytes from a selected immune donor mouse were fused with the myeloma cell line NS-1 using polyethylene glycol. Hybrid cells were selected through hypoxanthine-aminopterin-thymidine (HAT) supplemented medium, and culture supernatants were screened for ES antibody using ^{3}HES (in conjunction with dextran-charcoal separation of bound and free). Hybridomas from positive wells were cloned twice by limiting dilution, and a specific clone secreting monoclonal antibody to ES was grown up in culture, and injected into pristane primed Balb/c mice to produce ascites fluid containing a high concentration of antibody. The ascites fluid was collected and stored frozen at -20°C until required. Cells from the selected ES antibody secreting clone were also cryopreserved.

Oestrone sulphate radioimmunoassay

ES was measured in samples of whole milk by radioimmunoassay (RIA). The antibody used was the ES monoclonal in the form of crude ascites fluid, diluted in assay buffer (0.1M phosphate buffer containing 0.9% sodium chloride, 0.1% gelatin and 0.1% sodium azide, pH 7.0) to bind 50% of the added ^{3}HES tracer. The ES standards for the RIA were prepared in milk pooled from non-pregnant cows in which the endogenous concentration of ES was undetectable. A 100 µl milk sample or 100µl of ES standard (0 to 1000 pg), 100 µl of antibody and 10,000 cpm of 6,7 -^{3}H estrone sulphate, ammonium salt, in 100 µl assay buffer were incubated together overnight at 4°C. One ml of dextran-coated charcoal (0.075% charcoal, Norit A and 0.025% dextran T-70 in assay buffer) at 4°C was added, and the tubes centrifuged for 10 mins at 3000g, after 15 min. incubation at 4°C. The supernatant fraction containing antibody bound counts was decanted into scintillation vials, scintillation fluid added and the vials counted in a liquid scintillation spectrometer. A standard curve of the logit of percentage counts bound (B/B_o) versus the logarithm of ES standard concentration was plotted and the ES concentration in milk samples calculated by interpolation.

Milk samples

Whole milk samples were taken from 6 herds of Jersey and Friesian dairy cows which received a sole diet of ryegrass/clover pasture grazed in situ. Milk samples from pregnant cows were taken from cows which had undergone artificial insemination and

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in which pregnancy was confirmed by manual palpation of uterine contents at intervals throughout pregnancy, and/or by reference to calving records. The day of pregnancy was calculated from herd breeding records. Milk samples from non-pregnant cows were taken from a group of 35 cows which had not been inseminated. Collected milk samples were stored frozen until required.

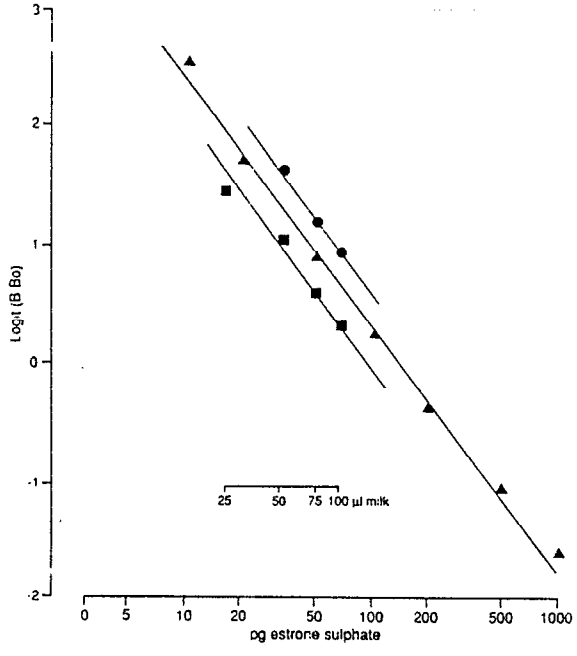
RESULTS

Characteristics of the ES monoclonal antibody and the ES RIA

Isotype analysis of the ES monoclonal antibody indicated it to be of the type IgG1. Specificity studies showed that relative to ES (taken as 100% cross-reaction), the monoclonal antibody cross-reacted 31% with oestrone; 0.2 to 0.4% with 17ß-oestradiol, 17α-oestradiol and testosterone and <0.1% with oestriol, 16-epioestriol, d-equilenin and progesterone. (Cross-reaction was defined as 100x(pg ES displacing 50% of ^{3}HES/pg steroid displacing 50% of ^{3}HES)). Logit-log plots of ES standards produced a linear response (Fig 1); the associated regression equation being logit (Y) = 4.5-2.1 log (X), r=-0.998, P<0.001. The sensitivity of the assay, calculated as the amount of ES causing a displacement of two standard deviations below the binding of the zero standard, ranged from 28 to 54 pg/ml. Lines parallel to the standard curve were obtained when different volumes (25 to 100µl) of 2 milk samples with high endogenous ES concentrations were assayed, and the results plotted as logit (B/B_o) vs log (volume) (Fig 1). The intra- and inter-assay coefficients of variation were <11%.

FIGURE 1 Logit-log plots of ES standards (▲ - ▲) and volumes of milk sampled from 2 pregnant cows (● - ●, ■ - ■).

Values for ES standards are means of 10 separate assays. 25 to 100 µl volumes of milk from the 2 pregnant cows were assayed, and the values plotted are means of triplicates. The coefficient of variation associated with each mean value is <7%.



Oestrone sulphate concentrations in milk during pregnancy

Fig 2 shows mean ± s.e.m. concentrations of ES in whole milk sampled from 20 non-pregnant cows, and 20 pregnant cows sampled weekly during pregnancy. In the non-pregnant cows, ES concentrations in milk ranged from non-detectable to 110 pg/ml, with a mean ± s.e.m. value of 58 ± 5 pg/ml. By 40-60 days of pregnancy, the mean concentration of ES (99 ± 10 pg/ml) in milk was already significantly higher than that found in the non-pregnant cows (P<0.001, Student's t-test). Mean concentrations of ES increased progressively from 40-60 days of pregnancy to plateau at approximately 1000 pg/ml after about 160 days. There was, however, considerable variation between individual cows in the profiles of ES concentrations in milk during pregnancy. In particular, in some pregnant cows ES concentrations in milk rose within 60 days of pregnancy to levels greater than the highest values found in milk from the non-pregnant cows. In other cows, this did not occur before day 100 of pregnancy. ES concentrations in the milk of individual pregnant animals could also fluctuate markedly from week to week. Examination of the ranges of ES concentrations found in milk indicated that 96% of cows ≥ 120 days pregnant had ES concentrations of 125 pg/ml or more (Table 1). Milk of all non-pregnant cows sampled had ES concentrations <125 pg/ml, as did 46% of cows < 120 days pregnant. The concentration of ES in milk sampled 120 days or more after mating/insemination can therefore provide an accurate indication of pregnancy status.

FIGURE 2 Oestrone sulphate concentrations in milk from non-pregnant cows and from cows sampled weekly during pregnancy.

Values are mean ± s.e.m. for 20 cows.

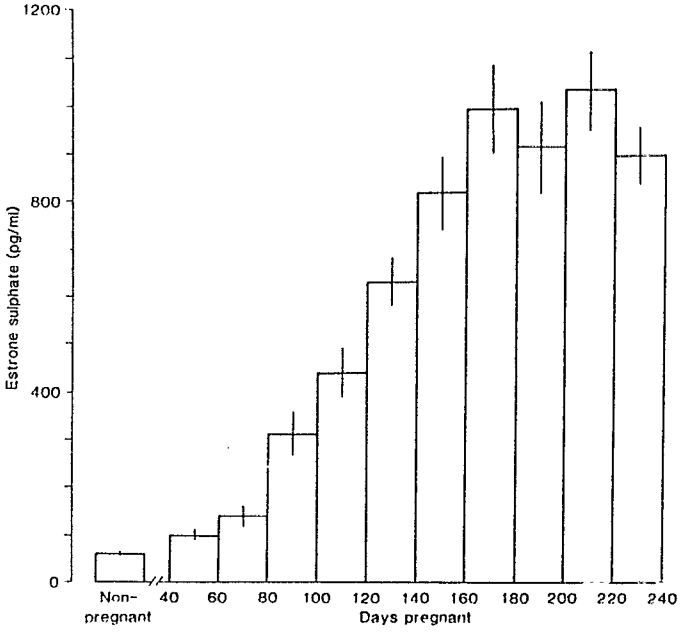


TABLE 1 Distribution of oestrone sulphate (ES) concentrations in milk from non-pregnant cows, and cows more than or less than 120 days pregnant.

Status	No. of cows with milk ES concs of:	
	<125 pg/ml	≥125 pg/ml
Non-pregnant	37 (100%)	0 (0%)
<120 days pregnant	87 (46%)	103 (54%)
≥120 days pregnant	14 (4%)	326 (96%)

DISCUSSION

Although previous studies have described radioimmunoassays to measure ES concentrations in cows' milk, the samples have been required to be defatted, or subjected to hydrolysis and solvent extraction prior to radioimmunoassay (Heap & Hamon, 1979; Holdsworth & Chaplin 1982; Holdsworth *et al.*, 1982; McCaughey *et al.*, 1982). In this study, whole milk was used in the radioimmunoassay thereby providing a simpler assay which may be more readily applicable to the routine assay of large numbers of samples. Polyclonal antisera were also used in the other assays, whereas this study utilised a monoclonal antibody. While using a monoclonal antibody may offer no advantage in a radioimmunoassay, its potential unlimited supply can make it a more useful diagnostic reagent than a polyclonal antibody, the characteristics of which may change with each collection of antibody. The generated monoclonal antibody showed good specificity for ES, except for the 31% cross-reaction with oestrone. Potential cross-reaction with oestrone should not be a problem in measuring ES concentrations in cows' milk. The concentration of oestrone in the maternal circulation of cows during pregnancy is <10% that of ES, and is positively correlated with the concentration of ES (Robertson & King, 1979). In this study the concentrations of ES present in milk of non-pregnant and pregnant New Zealand cows, and the ES profiles during pregnancy were very similar to the results of overseas studies (Hamon *et al.*, 1981; Heap & Hamon 1979; Heap *et al.*, 1983; Holdsworth *et al.*, 1982; McCaughey *et al.*, 1982; Power *et al.*, 1985). These studies also found that the concentration of ES present in milk obtained at least 100 days after mating/insemination could be used to discriminate between non-pregnant and pregnant cows.

In summary, the present study demonstrates for New Zealand dairy cows that the concentration of ES in whole milk obtained at least 120 days after mating/insemination can provide an accurate indication of pregnancy status, as samples from 96% of cows >120 days pregnant had milk ES concentrations of >125 pg/ml (Table 1). In milk samples obtained before 120 days, pregnancy may be confirmed if ES concentrations are >125 pg/ml. However, if values are <125 pg/ml an accurate diagnosis cannot be made, as 46% of pregnant cows sampled before 120 days and all non-pregnant cows sampled had milk ES concentrations <125 pg/ml.

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