The effect of once daily milking on concentrations and yields of plasminogen, plasmin and other whey proteins

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ABSTRACT

The objective of this study was to examine the effects of once daily milking (ODM) on yields of plasminogen, plasmin and other whey proteins and to investigate the possible role of plasmin in mammary gland involution. Twelve multiparous Friesian cows in the ninth month of lactation were placed on a ODM regimen for 7 days. Milk yields were determined and milk sampled for assay on the last day of twice daily milking (TDM) (day 1 of trial), days 3 and 7 of ODM (d4 & d8) and again 7 days after return to TDM (d15). Data are expressed as least-square means for d1 (TDM) vs d4 (ODM) vs d8 (ODM) vs d15 (TDM) together with pooled SE (PSE). Once daily milking was associated with a significant (P<0.05) reduction in yields of milk, lactose, fat and total protein. The concentration of plasminogen increased during ODM (0.92 vs 1.52 vs 1.37 vs 0.95, PSE = 0.06 µg/ml, P<0.01) but the yield of plasminogen was only marginally influenced by ODM (12.6 vs 14.8 vs 13.3 vs 10.5, PSE = 0.5 mg/d, P<0.05). The concentration of plasmin remained constant during the experiment (0.09 vs 0.10 vs 0.09 vs 0.08, PSE = 0.01 µg/ml, P>0.05) while the yield of plasmin decreased with ODM and did not recover on return to TDM (1.2 vs 1.0 vs 0.8 vs 0.8, PSE = 0.05 mg/d, P<0.001). The yield ratio of plasmin:plasminogen decreased during once daily milking (0.10 vs 0.07 vs 0.07 vs 0.09, PSE = 0.006, P<0.01). Once daily milking was also associated with a reduction in yield of ß-lactoglobulin but not of α-lactalbumin. The increased concentrations of plasminogen in milk during ODM suggest an opportunity for the involvement of proteases in mammary gland involution but their role is unclear given that milk plasmin yields decline during ODM.

Keywords: plasminogen, plasmin, α-lactalbumin, ß-lactoglobulin, once daily milking.

INTRODUCTION

Plasmin (EC 3.4.21.7) is a blood enzyme which is also the main proteolytic enzyme in bovine milk. Its proteolytic activity is responsible for the hydrolysis of the ß-casein to γ-casein and proteose-peptones (Eigel and Keenan, 1978; Greenberg and Groves, 1984) and the extent of this proteolysis affects the coagulation properties of milk (Bastain et al., 1991). Plasmin has been implicated in the involution process because it degrades milk proteins and it may have a role in tissue remodelling upon cessation of milking (Hedkvist et al., 1989; Politis et al., 1989a).

Plasmin concentration is low in normal milk and most of the enzyme exists as its zymogen, plasminogen. Conversion of plasminogen to plasmin requires specific peptide bond cleavage by plasminogen activators (PA). Two plasminogen activators, urokinase-PA and tissue-PA, have been found in the mammary gland and milk (Ossowski et al., 1979; Busso et al., 1989). Blood serum-derived inhibitors are also found in milk and could be responsible for inhibiting plasmin activity (Korycka-Dahl et al,. 1983). In addition ß-lactoglobulin may have an inhibiting effect on plasmin activity (Humbert and Alais, 1979; Grufferty and Fox, 1988a, 1988b).

The mechanism by which plasminogen and plasmin pass from blood into milk is unknown. It has been assumed that the increases in plasmin and plasminogen-derived activities in late- compared to early-lactation milk are due to increased permeability of the blood vessels and secretory epithelium, allowing more plasminogen to pass into the gland (Schaar and Funke, 1986). However, plasmin activity may also be affected by changes in concentrations of activators and inhibitors which affect the conversion of plasminogen to plasmin in milk. Plasmin activity in milk increases towards the end of lactation (Richardson, 1983; Politis et al., 1989a, 1989b; Benslimane et al., 1990; Politis et al., 1990; Bastain et al., 1991), during mastitis (Schaar and Funke, 1986; Kaartinen et al., 1988; Saeman et al., 1988; Politis et al., 1989a) and in milk from older cows (Mattila et al., 1986; Bastain et al., 1991).

The objective of this study was to determine the effect of ODM on concentrations and yields of plasminogen, plasmin and other whey proteins and to investigate the possible role of plasmin in the initial steps of mammary gland involution

MATERIALS AND METHODS

Twelve Friesian cows in the ninth month of their second or third lactation with historical somatic cell counts of less than 225,000 cells/ml and a mean production index of 125 (range 107-142) were used. Throughout the experimental period they grazed mixed ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) pastures with the main herd. A switch back experimental design (Arnold, 1981) was used, in which the cows were milked twice daily (TDM, days -1 to 1 of the experiment), once daily for 7 days (ODM, days

2-8) and then returned to twice daily milking for 7 days (TDM, days 9-15).

Milk samples were collected for the determination of concentrations of fat, total protein, lactose, plasminogen and plasmin on days -1, 0, 1 (TDM), days 4 and 8 (ODM) and day 15 (TDM). Alpha-lactalbumin and ß-lactoglobulin were assayed in milk collected on days 0 (TDM), 8 (ODM), and 15 (TDM). Milk yield was estimated by an in-line metering system (Westphalia, Metatron, Germany) and milk samples were analysed for fat, total protein and lactose using a Milkoscan A/B 140 (A/S N. Foss Electric, Denmark).

Assays

Milk samples were placed on ice after collection and skim milk was prepared by centrifugation at 3,000 g for 20 min at 4°C. Plasminogen and plasmin were dissociated from casein micelles by incubation of skim milk with 75 mM ε-amino-n-caproic acid (A2504, Sigma Chemical Co., St. Louis, MO) for 2h. Bacterial growth was controlled by the addition of 0.1% NaN_3. Whey was prepared by ultracentrifugation of the skim milk at 100,000 g for 1h at 4°C. The whey fraction was stored at -20°C prior to analysis.

Plasminogen-derived and plasmin activities were determined in whey samples using the method described by Korycka-Dahl et al. (1983) as modified for use with a Cobas Fara II autoanalyser (Hoffman-La Roche, Switzerland). Total plasmin plus plasminogen-derived activity (defined as the proteolytic activity that was activated by urokinase) was determined in a reaction mixture (300 µl) of 50 mM Tris-HCl buffer (pH 7.4) containing 110 mM NaCl, 0.6 mM H-D-Valyl-L-leucyl-L-lysine-p-nitroanilide dihydrochloride (V 7127, Sigma Chemical Co.), 0.17% bSA, 1.5 mM ε-amino-n-caproic acid and 10 µl of whey or standard solution (plasminogen, P 6144, Sigma Chemical Co.). Forty microliters of urokinase (U 8627, Sigma Chemical Co.) solution (20-48 Ploug units) was added at time zero. The reaction mixture was incubated at 37°C for 10 min and absorbance measured at 2.5 min intervals for 0.75h. Each sample was replicated twice. Plasmin (P 7911, Sigma Chemical Co.) was measured in a similar reaction but without the addition of urokinase and using 25 µl of whey or standard solution. Plasmin concentrations were calculated directly and plasminogen-derived activity was then determined by difference.

Alpha-lactalbumin and ß-lactoglobulin concentrations were determined by fast protein liquid chromatography as described by Andrews et al. (1985). A 100 µl sample of whey was passed through a 0.2 µm filter, diluted 1:1 or 1:3 with 20 mM Tris-HCl buffer, pH 7.0, and applied to a Mono Q HR 5/5 column (strong anion exchange). The column was eluted with a 0-0.35 M NaCl salt gradient in the buffer at a flow rate of 1 ml/min over a period of 30 min.

Statistical Methods

Yield of milk and its components were analysed using an analysis of variance for a switch back design (Arnold, 1981). Yields and concentrations during the treatment (ODM) period were compared to those during the pre- and post-treatment periods (TDM). Yields and concentrations from individual days in the pre-treatment period were averaged

and designated day 1 of the trial. Milk components were analysed in milk from all twelve cows, with the exception of α-lactalbumin and ß-lactoglobulin, which were analysed in only 8 cows selected at random from the experimental group. The model used to analyse yield of milk and its components was:

$$Y_{ij} = \mu + p_i + c_j + e_{ij}$$

where Y_{ij} = observation on the jth animal in the ith period;

μ = population mean;

p_i = effect of the ith period (i=1..3);

c_j = effect of the jth cow (j=1..12 or 1..8); and

e_{ij} = a random residual unique to the jth cow in the ith period.

This model allowed the testing of the contrasts d1 (TDM) vs d4 (ODM) vs d15 (TDM) or d1 vs d8 (ODM) vs d15, but not d4 vs d8 (within the ODM period).

RESULTS

The concentrations and yields of individual components in the milk over the experimental period are presented in Tables 1 and 2 respectively. Relative to day 1 (d1) the concentration of lactose decreased (P<0.05) by the seventh day (d8) of ODM, while the concentrations of fat increased (P<0.05). Total protein concentration also increased during once daily milking (d4, P<0.05) but did not differ significantly (P>0.05) at d8 from that at d1. After seven days of ODM, milk, lactose, fat and total protein yields decreased (P<0.05) by 27%, 29%, 21% and 22% respectively relative to pre-treatment yields. The yields then recovered to some extent with the resumption of TDM (Table 2).

The concentrations of milk-specific proteins, namely α-lactalbumin and ß-lactoglobulin, increased (P<0.05) by 24% and 15% respectively after seven days of ODM (d8 vs d1). However, the yield of α-lactalbumin was unaffected whereas the yield of ß-lactoglobulin declined after seven days of ODM (Table 2).

Plasminogen concentration increased (P<0.05) by 65% on d4 and by 49% on d8, relative to that on d1. Upon return to TDM, plasminogen concentration returned to pre-treatment concentrations (Table 1). Plasminogen yield increased (P<0.05) within 3 days of ODM (d4) but by the seventh day (d8) the yield was not significantly (P>0.05) different from pre-treatment yield (Table 2). In contrast, the concentration of plasmin did not change significantly (P>0.05) during ODM compared to TDM (Table 1), and thus the yield of plasmin fell by 48% (P<0.05) by the seventh day of ODM (Table 2). The plasmin:plasminogen ratio declined 36% by d4 of the trial relative to d1 (P<0.05). By the seventh day of ODM the ratio remained the same, and on return to TDM the ratio nearly recovered to pre-treatment values.

DISCUSSION

The objective of this study was to examine the changes in concentrations and yields of plasminogen and plasmin during ODM, and to investigate the possible role of plasmin in mammary gland involution.

The decreases in milk, lactose, fat and total protein yields during ODM are similar to losses reported by Bryant

TABLE 1: Concentration of milk components (least-square mean ± se) from twelve Friesian cows in the ninth month of their second or third lactation. Cows were milked twice daily (day 1 of trial), once daily for seven days (sampled days 4 & 8) and then returned to twice daily milking for seven days (sampled day 15).

Concentration	n	Day 1	Day 4	Day 8	Day 15
Lactose (mg/ml)	12	48.2 ± 0.2[aA]	46.1 ± 0.3[b]	45.7 ± 0.2[B]	48.8 ± 0.2[aC]
Total Protein (mg/ml)	12	39.3 ± 0.5[aA]	41.9 ± 0.5[b]	40.2 ± 0.5[A]	40.2 ± 0.5[abA]
Fat (mg/ml)	12	47.9 ± 1.6[aA]	52.9 ± 1.4[b]	58.0 ± 1.7[B]	53.9 ± 1.6[bC]
α-lactalbumin (mg/ml)	8	1.11 ± 0.08[A]	N/A	1.38 ± 0.08[B]	1.21 ± 0.09[B]
ß-lactoglobulin (mg/ml)	8	5.87 ± 0.26[A]	N/A	6.74 ± 0.26[B]	6.04 ± 0.29[AB]
Plasminogen (μg/ml)	12	0.92 ± 0.05[aA]	1.52 ± 0.08[b]	1.37 ± 0.06[B]	0.95 ± 0.06[aA]
Plasmin (μg/ml)	12	0.09 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.08 ± 0.01

[a,b,c] means in same row with superscript letters in common do not differ significantly (P>0.05) (Day 1 vs Day 4 vs Day 15)

[A,B,C] means in same row with superscript letters in common do not differ significantly (P>0.05) (Day 1 vs Day 8 vs Day 15)

N/A — not assayed

n — number of cows (see "Statistical Methods")

TABLE 2: Yields of milk and its components (least-square mean ± se) from twelve Friesian cows in the ninth month of their second or third lactation. Cows were milked twice daily (day 1 of trial), once daily for seven days (sampled days 4 & 8) and then returned to twice daily milking for seven days (sampled day 15).

Yield	n	Day 1	Day 4	Day 8	Day 15
Milk (kg/day)	12	14.50 ± 0.23[aA]	10.53 ± 0.23[b]	10.65 ± 0.23[B]	11.88 ± 0.23[cC]
Lactose (kg/day)	12	0.698 ± 0.013[aA]	0.485 ± 0.013[b]	0.499 ± 0.013[B]	0.579 ± 0.013[cC]
Total Protein (kg/day)	12	0.559 ± 0.009[aA]	0.437 ± 0.008[b]	0.437 ± 0.009[B]	0.472 ± 0.009[cC]
Fat (kg/day)	12	0.658 ± 0.014[aA]	0.554 ± 0.014[b]	0.512 ± 0.014[B]	0.620 ± 0.014[cC]
α-lactalbumin (g/day)	8	13.95 ± 0.68	N/A	12.65 ± 0.68	12.39 ± 0.75
ß-lactoglobulin (g/day)	8	74.56 ± 2.03[A]	N/A	60.15 ± 2.03[B]	62.79 ± 2.24[B]
Plasminogen (mg/day)	12	12.58 ± 0.45[aA]	14.76 ± 0.63[b]	13.30 ± 0.48[A]	10.50 ± 0.48[cB]
Plasmin (mg/day)	12	1.17 ± 0.05[aA]	0.95 ± 0.05[b]	0.80 ± 0.05[b]	0.82 ± 0.05[bB]
Plasmin: Plasminogen Yield Ratio	12	0.10 ± 0.01[aA]	0.07 ± 0.01[b]	0.07 ± 0.01[B]	0.09 ± 0.01[abAB]

[a,b,c] means in same row with superscript letters in common do not differ significantly (P>0.05) (Day 1 vs Day 4 vs Day 15)

[A,B,C] means in same row with superscript letters in common do not differ significantly (P>0.05) (Day 1 vs Day 8 vs Day 15)

N/A — not assayed

n — number of cows (see "Statistical Methods")

(1979), Woolford *et al.* (1985) and Carruthers and Copeman (1990) for cows in the declining phase of lactation. The decrease in yield of milk-specific components, namely α-lactalbumin and ß-lactoglobulin, reflects a general decrease in the secretory activity of the mammary gland tissue following an extended milking interval (Wheelock *et al.*, 1965). The difference in magnitude of the decrease probably reflects both differences in inhibition of secretion and variation in the rate of removal of the individual components from the mammary gland (Linzell and Peaker, 1971; Lascelles and Lee, 1978; Wilde *et al.*, 1987, 1988).

Plasminogen yield had increased by d4, however by d8 the yield was not significantly different from the pre-treatment yield. The transient increase in yield of plasminogen must have been due to an increased plasminogen transfer from interstitial fluid into milk, as plasminogen found in milk is identical to blood plasminogen (Rollema *et al.*, 1981; Humbert *et al.*, 1990) and no synthesis is thought to occur in the mammary gland (Grufferty and Fox, 1988a). However, this change was transient and the opportunity for its activation to plasmin, and hence its role in causing milk yield losses during ODM, is unclear. Despite the rise in plasminogen

yield, plasmin yield decreased during ODM and did not recover on return to TDM. As a result there was a marked decline in the plasmin:plasminogen yield ratio. This indicates that the rate of activation of plasminogen was reduced by inhibitors or that the rate at which plasmin breaks down was increased.

The decline in plasmin yield during ODM, despite a transient rise in plasminogen yield, suggests that plasmin is unlikely to have a major role in the accelerated involution assumed to be associated with ODM (Wilde and Knight, 1990). This contrasts with the results of Politis *et al.* (1989a; 1990) in which plasmin concentrations increased 72h after complete cessation of milking and may reflect differences between ODM and dried-off cows in the extent of mammary gland changes.

CONCLUSIONS

Once daily milking was associated with a decline in milk, lactose, fat and total protein yields. There was a transient increase in plasminogen yield, but an associated decreased yield of plasmin. During ODM the plasmin:

plasminogen ratio was decreased by 36% and this suggests plasmin does not play a significant role in mammary gland involution, at least during short periods of ODM. A further study, in which the milking interval was extended further than 24h, may allow the role of plasmin in mammary gland involution to be investigated further.

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