Effect of Once Daily Milking and Concurrent Somatotropin on Mammary Tight Junction Permeability and Yield of Cows

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Six pairs of monozygous Friesian twin cows during late lactation were used to assess the effect of once daily milking and bST treatment on yields and tight junction permeability of mammary epithelial cells. During the first 7 d, all cows were milked twice daily; on d 8 through d 21, all cows were milked once daily, but one cow of each twin pair was treated daily with 20 mg of bST on d 13 through 21; and, finally, during d 22 through 28, all cows were again milked twice daily. Once daily milking, a common management practice in New Zealand, resulted in a small (7%) but significant decrease in milk yield. Treatment with bST increased milk yield by 19%, thereby exceeding the milk yield loss from once daily milking. The integrity of mammary tight junctions was assessed indirectly by measuring concentrations of plasma lactose and milk BSA. Once daily milking temporarily disrupted tight junction integrity, based on a 4- to 5-fold increase in plasma lactose and a 42 to 55% increase in the concentrations of milk BSA. In the present study, bST did not affect the permeability of mammary tight junctions.

(Key words: tight junction, mammary gland, milking interval, milk)

Abbreviation key: FMGC = functional mammary gland capacity, ODM = once daily milking, PVP = polyvinylpyrrolidone, SED = standard error of difference, TDM = twice daily milking, TJ = tight junctions.

INTRODUCTION

Once daily milking (ODM) is well established among New Zealand dairy farmers as a management tool and as a means to lower labor costs. Unfortunately, ODM substantially reduces milk yield of cows (4, 5, 8), sheep (12), and goats (18). Although the exact cause of such loss is not known, unilateral ODM in goats (18), sheep (12), and cows (K. Stelwagen and C. H. Knight, 1993, unpublished results) decreased milk yield by, respectively, 26, 20, and 40% in the ODM glands only, suggesting a local control mechanism. Indeed, evidence exists for the presence of a local intramammary inhibitor of milk secretion (19). For goats, milk secretion and mammary blood flow began to decrease approximately 21 h following milking, i.e., when disruption of mammary tight junctions (TJ) was initiated, based on a rise in lactose concentrations in plasma and a decrease in potential difference across the mammary gland (15). These data suggest that loss of TJ integrity may be a factor involved in the yield loss associated with ODM. The TJ are small extracellular structures that form a tight seal around adjacent secretory cells near the apical domain, hence preventing blood components from crossing into milk and vice versa.

For cows on ODM, the percentage of lactose in milk was significantly lower than that...
in the milk of twice daily milked (TDM) cows (4, 5, 8). Because lactose is the major osmoregulatory component in milk, a decrease in its content would probably be caused by lactose leaking into the blood via disrupted TJ. In contrast, when cows were treated with bST, the lactose content in milk increased again until treatment ceased, at which point the lactose content decreased (4), implying that bST may have a positive effect on TJ maintenance.

Although evidence is ample that exogenous bST increases milk yield in TDM cows (10), only one study exists in which bST was used during ODM (4). In that study, ODM reduced milk yield by approximately 17%, but bST increased milk yield by 13%, almost overcoming the loss caused by ODM.

The aim of the present study was twofold: first, to investigate the effect of ODM and exogenous bST during ODM on TJ patency and, second, to study the milk production response to bST during ODM.

MATERIALS AND METHODS

Cows and Management

Six pairs of multiparous, pregnant, monozygous twin Friesian cows of the Dairying Research Corporation's Dairy Number 1 twin herd were used. All cows were in late lactation (213.4 ± 16.7 d) at the start of the experiment. Cows were normally milked at 0700 and 1500 h during TDM and at 0700 h during ODM. Throughout the experiment, the cows were fed pasture and supplemented with silage for ad libitum intake, but no concentrates were fed. Cows had ad libitum access to water.

Experimental Design

The experiment lasted 28 d and was divided into four periods. Period 1 consisted of the first 7 d, during which all cows were milked twice daily. During d 8 through 13 (period 2), all cows were milked once daily. In period 3, d 14 through 21, ODM continued, but 1 cow of each twin set was treated intramuscularly with 20 mg/d of recombinant bST (Eli Lilly and Company, Greenfield, IN). Cows were treated every day immediately before the morning milking. The untreated twin sister served as a control. During the final 7 d (period 4), all cows were again on TDM.

Milk weights were recorded at every milking. In each period, milk samples for fat, protein, lactose, and BSA were taken during two morning and two afternoon milkings (TDM) or during two morning milkings only (ODM). Samples for SCC measurements were taken on separate occasions during an a.m. and p.m. milking every week. To determine concentrations of plasma lactose, blood samples (10 ml) were obtained from the coccygeal vein by venipuncture on 3 d/wk immediately prior to the morning milking.

Immediately following the last day of the experiment, functional mammary gland capacity (FMGC) was determined following a 40-h milking interval, as described by Davis and Hughson (6). Alveolar milk was collected by machine milking following intraperineal injection of 10 IU of oxytocin (Intervet Pty. Ltd., Lane Cove, New South Wales 2066, Australia) 10 min after the normal milking.

Chemical Analyses

Milk Composition. Milk fat, protein, and lactose were analyzed by infrared analysis (Milko Scan 133B; Foss Electric, Hillerød, Denmark), and SCC were determined using a cell counter (Fossomatic 450; Foss Electric).

BSA in Milk. Fresh milk samples were defatted by centrifugation for 15 min at 490 x g, and the skim milk fraction was frozen at -20°C until analyzed by ELISA. Prior to analysis, skim milk samples were thawed and diluted 5000-fold with ELISA diluent [.1% polyvinylpyrrolidone (PVP; Serva Feinbiochemica GmbH, Heidelberg, Germany) prepared in .1 M PBS, pH 7.5, containing .05% Tween 20]. The ELISA was carried out with two 96-well polystyrene microtitre plates, referred to as mixing plate and ELISA plate. To each well of the mixing plate, 100-pl skim milk samples or BSA standards (Sigma Chemical Co., St. Louis, MO) were incubated overnight at 4°C with 100 µl of anti-rabbit BSA (DAKO Corporation, Carpintera, CA; final dilution of 1:50,000 in ELISA diluent). The ELISA plate was prepared as follows: to each well of the mixing plate, 100-µl skim milk samples or BSA standards (Sigma Chemical Co., St. Louis, MO) were incubated overnight at 4°C with 100 µl of anti-rabbit BSA (DAKO Corporation, Carpintera, CA; final dilution of 1:50,000 in ELISA diluent). The ELISA plate was prepared as follows: to each well, 100 µl of coating solution (1 µg/ml of BSA in .1 M NaHCO₃; pH 9.6) were added, and the samples were incubated overnight at 4°C. Next the wells were washed three times with .1 M PBS (pH 7.5, containing .05% Tween 20).
Tween 20), and 200 μl of ELISA diluent were added as a blocking agent. Following 30 min of incubation at 20°C, the wells were washed twice as previously. Then 100 μl of the mixture from the mixing plate wells were added, and samples were incubated at 20°C for exactly 60 min. Wells were washed five times as before with 100 μl of donkey anti-rabbit IgG peroxidase conjugate (Amersham Life Sciences, Auckland, New Zealand) diluted 1:2000 in ELISA diluent and then incubated for 30 min at 20°C. Then wells were washed six times as before, after which 100 μl of substrate solution (0.1% 2',2'-azino-diethylbenzothiazoline (Sigma Chemical Co.) in citrate-phosphate buffer, pH 4.0, containing 0.003% w/vol, H2O2) were added to each well. Following a final incubation of 30 min at 20°C, absorbance was measured at 405 nm, using 490 nm as a reference wavelength. Parallelism was confirmed by plotting dilutions of three randomly selected samples alongside the standard curve. Bovine IgG (Chemicon International, Temecula, CA), α-lactalbumin, β-lactoglobulin, lactoferrin, α-casein, β-casein, and κ-casein (Sigma Chemical Co.) did not crossreact with BSA, recovery of an added mass of lactose was 113%, and intraassay and interassay coefficients of variation were <10%. To avoid confounding from interassay variation, all samples of a twin pair were run in the same assay.

Lactose in Plasma. Blood was collected in heparinized vacutainers, and centrifuged for 15 min at 490 x g; plasma was collected and stored at -20°C until analyzed by an enzymatic assay. Prior to analysis, each sample was deproteinized with perchloric acid, using a modification of the method by Arthur et al. (2). Briefly, 500 μl of plasma were mixed with 1 ml of 1 M perchloric acid. After samples stood for 5 min and were centrifuged for 30 min at 2000 x g, 4°C, 900 μl of supernatant were mixed with 300 μl of neutralizing buffer (containing equal volumes of 4 M KOH and 1 M K2PO4, pH 7.0). Following standing and centrifugation as before, the supernatant was either frozen or used immediately for lactose analyses. All solutions were kept at 4°C. Lactose (BDH Chemicals Ltd., Palmerston North, New Zealand) standards for the assay were subjected to the same deproteinization procedure as the plasma samples. The enzymatic assay is based on two reactions, one measuring galactose and the other measuring lactose and galactose; the difference between the two provided a measurement of lactose concentration. Deproteinized samples and standards were loaded (100 μl) onto 96-well polystyrene microtiter plates and mixed with an equal volume of enzyme reagent A (to measure galactose) or B (to measure lactose and galactose). Reagent A consists of 1 ml of MOPS [3-(N-morpholino)-propanesulfonic acid; Sigma Chemical Co.] buffer (0.5 M MOPS and 1 M NaOH to pH 7), 0.5 mg of thio-NAD (Sigma Chemical Co.), 10 μl of MgSO4 in H2O, and 4.5 μl of galactose dehydrogenase-S (Boehringer Mannheim Ltd., Auckland, New Zealand; 50 U/ml of 3.2 M ammonium sulfate, pH 6). Reagent B consists of 1 ml of reagent A and 15 μl of β-galactosidase (Boehringer Mannheim Ltd.), 1500 U/ml of 3.2 M ammonium sulfate, pH 6). Mixtures were incubated at 20°C for 60 min after which absorbance was measured at 405 nm, using 490 nm as a reference wavelength. The sensitivity of the assay was 18 μM (95% confidence interval), and recovery of an added mass of lactose was 116%. Intraassay and interassay coefficients of variation were <10% and <20%, respectively. To avoid confounding from the interassay variation, statistical analyses of all samples of a twin pair were run in the same assay.

Statistical Analyses

Milk fat, protein, lactose, BSA, and SCC during TDM (periods 1 and 4) are presented as weighted averages, i.e., corrected for morning and afternoon milk yields.

Statistical analyses were carried out using Minitab (11). Differences between twin sisters and between periods within animal were evaluated by paired t test and were considered to be significant at P < 0.05. Standard errors presented in Table 1, and Figures 1 to 4 present standard errors of the difference (SED) between paired means.

RESULTS

Daily milk yields are shown in Figure 1, and yield, composition, and SCC data for each period are presented in Table 1. During period 2, one cow developed clinical mastitis and was treated successfully with antibiotics, but
TABLE 1. Performance of monozygous twins during periods of twice (TDM) and once daily milking (ODM) and during bST treatment (n = 6 per treatment group).

<table>
<thead>
<tr>
<th></th>
<th>Period 1</th>
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<th>Period 2</th>
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<th>Period 3</th>
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<td>SED</td>
<td>Control</td>
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<td>SED</td>
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<td>bST</td>
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<td>13.3</td>
<td>.5</td>
<td>12.3</td>
<td>12.4</td>
<td>.5</td>
<td>12.2</td>
<td>14.6</td>
<td>.7b</td>
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<td>Fat, %</td>
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<td>4.44</td>
<td>.07</td>
<td>4.81</td>
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<td>.08</td>
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<td>3.37</td>
<td>.04</td>
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<td>.06</td>
<td>3.69</td>
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<td>564.6</td>
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<td>1.6</td>
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<td>.5</td>
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<td>456.7</td>
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<td>539.7</td>
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*Control and bST groups per row within a period differ (P < .01).
*Control and bST groups per row within a period differ (P < .05).
*Control and bST groups per row within a period differ (P < .10).
*Control or bST groups within a row and between this and previous period differ (P < .01).
*Control or bST groups within a row and between this and previous period differ (P < .05).
*Control or bST groups within a row and between this and previous period differ (P < .10).

1Period 1, TDM (d 1 through 7); period 2, ODM (d 8 through 13); period 3, ODM with or without bST treatment (d 14 through 21); period 4, TDM (d 22 through 28).
2Change from one period to the next period.
Figure 1. Mean daily milk yield of monozygotic twin cows during twice (TDM) and once daily milking (ODM) and concurrent bST treatment. Control (○) and bST (*) profiles differ: *P < .05, †P < .10; bars represent standard errors of the difference.

Figure 2. Mean concentrations of BSA in milk in monozygotic twin cows during twice (TDM) and once daily milking (ODM) and concurrent bST treatment. Control (○) and bST (*) profiles differ: *P < .05, †P < .10; bars represent standard errors of the difference.

caus[ed a rather high SCC for the bST group in period 2. The data of this cow and her sister were excluded from the analysis of other parameters for period 2. With ODM, milk yield decreased by 6.8 to 7.5% (period 2) compared with the yield of the previous TDM period (period 1). However, during period 3, bST increased milk yield by 19.7 (compared with yield of control cows) or 18.1% (compared with yield in period 2) during ODM (Table 1; Figure 1). After bST treatment ceased, milk yield of the treated cows returned to that of the control group. However, compared with milk yields during TDM in period 1, milk yields of control cows during TDM in period 4 were still lower (P < .01), but milk yields of cows in the bST group (period 4) were not lower than those in period 1 (P > .10).

Changes in fat, protein, and lactose yield associated with bST treatment and with the switch from TDM to ODM reflect the corresponding changes in milk yield (Table 1). Milk protein percentage was increased by ODM, and the concentration of lactose tended to decrease with ODM (Table 1). During bST treatment (period 3), no difference in the concentrations of milk fat, protein, or lactose occurred between the bST-treated group and the control group; however, milk fat and lactose concentrations increased in the bST-treated group in period 3 from that during the pretreatment ODM period (period 2).

Concentrations of BSA in milk are presented in Figure 2. The ODM caused a rapid increase in milk BSA concentrations, from 42 to 56% (P < .01), which remained elevated in the control group throughout ODM (period 2 and 3). Concentrations of BSA in milk in period 3 were reduced (P < .05) by bST, but, following cessation of bST treatment, BSA increased again to those concentrations in the control group. Although BSA concentrations seemed to decline slightly during TDM in period 4, concentrations were still significantly (P < .01) higher than those during TDM in period 1. Total BSA output in the milk is depicted in Figure 3. The increase in BSA output (18 to 28%) was still significant (P < .01) as milking regimen changed from TDM to ODM; however, bST treatment had no effect on total BSA output in milk. As observed with the concentration of BSA in milk, total BSA in milk began to decline during subsequent TDM (period 4) but was still significantly higher than during the first period of TDM.

Results for lactose in plasma are shown in Figure 4. The concentration of lactose in plasma rose sharply (4.4- to 5.0-fold; P < .05) following the transition from TDM to ODM (period 1 vs. period 2), but, unlike milk BSA, had practically decreased to TDM concentrations 2 d later. Treatment with bST had no apparent effect on concentrations of lactose in plasma.

The FMGC (i.e., milk yield following a 40-h milking interval) and alveolar milk did not differ between the control and the bST
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Figure 3. Mean total BSA yield in milk of monozygotic twin cows during twice (TDM) and once daily milking (ODM) and concurrent bST treatment. Control (C) and bST (.) profiles differ: *P < .05; bars represent standard errors of the difference.

Figure 4. Mean concentrations of lactose in plasma of monozygotic twin cows during twice (TDM) and once daily milking (ODM) and concurrent bST treatment. Control (C) and bST (.) profiles differ: *P < .05; bars represent standard errors of the difference.

group (15.58 vs. 15.92 kg; SED .90; P > .10). In all periods, even during bST treatment (Table 1), FMGC was in excess of daily yield. Moreover, there was no apparent correlation between milk yield response to bST and FMGC (r = -.01; P > .10; n = 6). Compared with SCC in milk during period 4, the period preceding FMGC measurements, the SCC immediately following FMGC measurements were 10-fold higher (82,000 vs. 801,000 cells/ml; SED 267,000; P < .05).

DISCUSSION

The present experiment was carried out to determine the effect of ODM and concurrent bST treatment on the maintenance of TJ between mammary epithelial cells, and milk yield.

Consistent with results from studies of full lactation (5, 8) and from short, 1- to 2-wk studies [(3); K. Stelwagen, V. C. Farr, and S. R. Davis, 1993, unpublished results] in which cows were on ODM, in the present study, milk yield decreased slightly, but significantly with ODM. Causes of these yield losses are unknown. However, accelerated secretory cell loss is not likely to be a factor because, following short periods of ODM, milk yield returns to the preceding TDM level when cows are switched back to TDM [(3); K. Stelwagen, V. C. Farr and S. R. Davis, 1993, unpublished results]. However, there is strong support (19) for the presence of a factor in milk that inhibits secretion. Loss of TJ integrity may be another factor potentially involved in milk loss associated with ODM. Induced disruption of TJ reduced milk secretion in goats (13, 16) and cows (1), and we recently showed that, in goats, TJ integrity is compromised following approximately 21 h of milk accumulation, i.e., when the rate of milk secretion began to decline (15).

In the current study, we used concentrations of plasma lactose and milk BSA to assess TJ integrity indirectly. To assess TJ patency, we have previously used the measurement of the potential difference across the mammary gland (15) and the appearance of a dye (Evans Blue) in mammary lymph following administration of the dye via the teat during disruption of mammary TJ (16) induced by ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid and found that these measurements are in agreement with concomitant changes in the concentrations of lactose in plasma and BSA in milk. The transition from TDM to ODM substantially increased BSA in milk and plasma lactose concentrations, indicating disruption of mammary TJ. The 4- to 5-fold increase in plasma lactose was similar to that observed in goats (15). However, the plasma lactose and milk BSA patterns differed in that concentrations of lactose in plasma had returned almost to baseline levels 2 d later, but concentrations of BSA in milk remained elevated throughout ODM, confirming similar results from another study in which 24 cows were switched from TDM to ODM (K. Stelwa-
gen, V. C. Farr, and S. R. Davis, 1993, unpublished results). This unexpected pattern of BSA in milk cannot be attributed to decreased milk volume during ODM because total BSA production was significantly higher during ODM as well.

Because lactose is a much smaller molecule than BSA, the difference in the pattern of the concentrations of lactose in plasma and BSA in milk cannot be explained in terms of a gradual reformation of TJ, during which larger molecules would be excluded first. Instead, during the transition from ODM to TDM, TJ integrity seems to be severely disrupted, resulting in elevated concentrations of plasma lactose and milk BSA, but, within a few days of this transition, TJ integrity is apparently reestablished (i.e., plasma lactose concentrations return to TDM levels). However, the transfer of BSA into milk seems to be maintained after TJ become tight again and, thus, must be maintained through another mechanism. Although this mechanism is unknown, it is tempting to speculate that the mechanism might be related to an increased influx of lymphocytes crossing the TJ barrier in a diapedetic fashion. Goussault et al. (7) demonstrated that lymphocytes can synthesize and secrete serum albumin in vitro. Furthermore, ODM significantly increased milk SCC (presumably including lymphocytes) in cows (8). In the present study, milk SCC were also higher, albeit not significantly, during ODM. The implication of this increase of BSA in milk during ODM may be important because of its adverse effects on characteristics of milk processing.

In the present study, the transient disruption of mammary TJ during the transition from TDM to ODM, is consistent with our notion that loss of TJ integrity plays a role in the milk yield loss associated with ODM, but does not explain the sustained depression of milk yield during ODM. Traditionally, the TJ has been assumed to function as a barrier between the basolateral and apical sides of the mammary epithelial cell, modulating paracellular leakage. More recently, a second function of TJ has been established (14). In this latter function, also referred to as “fence” function, TJ serve as a boundary near the apical side of the cell, separating basolateral and apical membrane proteins and lipids (14) and as such are involved in cellular membrane organization. Both functions of the TJ are linked to the cytoskeleton of the cell (14), and, because the components of the cytoskeleton are involved in milk secretion (9), we envisage an essential role, albeit indirect, for TJ in the process of milk secretion. We therefore hypothesize that the barrier function of TJ is restored within a few days of the transition from TDM to ODM, explaining the return of the concentration of lactose in plasma to baseline levels, but the fence function remains impaired and is at least partly responsible for the sustained milk yield loss during ODM.

The milk yield response to bST in the present study was larger than that observed in the only other ODM study in which bST was used (4). In fact, the milk yields during bST treatment exceeded the yields during the preceding period of TDM, and the percentage increase is comparable with that obtained during TDM with bST (10). Although cisternal mammary capacity may be limiting during ODM (17), FMGC measurements in the current study revealed that the milk yield response to bST was not restricted by mammary capacity.

In the present study, as well as in a previous study (4), bST treatment reversed the milk loss associated with ODM. Possibly, bST down-regulates the expression of the putative inhibitor of milk secretion (19), and this possibility requires further investigation. In our study, we have examined the effect of bST on TJ permeability during ODM, because ODM decreases concentrations of lactose in milk (4, 5, 8), and bST treatment plus ODM increased the lactose content in milk (4). Because lactose, the predominant osmoregulator in milk, determines milk volume, changes in its concentration in the milk would seem to be due to lactose passing from milk into blood through impaired TJ. In agreement with results of these studies, milk lactose concentration was lower during ODM and increased during bST treatment. However, although BSA concentrations in milk were significantly lower during bST treatment, total BSA in milk was not affected by bST, suggesting that the lower BSA content in milk was due to the dilution caused by the extra milk in response to bST. Also, bST had no effect on plasma lactose concentrations. Thus, bST does not seem to affect the permeability (i.e., the barrier function) of TJ under these conditions.
In conclusion, compared with TDM, ODM reduced milk yield and temporarily disrupted TJ. Although bST treatment can compensate for the milk loss associated with ODM, it does not affect TJ permeability.

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