Effect of milking frequency on lactation persistency and mammary gland remodeling in mid-lactation cows¹

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ABSTRACT

The objective of this study was to evaluate the effect of milking frequency on milk production and composition, mammary cell proliferation, apoptosis, and gene expression. For this investigation, 10 Holstein cows that were being milked twice a day in mid lactation were selected. To study the effect of differential milking, 2 quarters were milked once daily and the other 2 were milked thrice daily for 8 wk. After that period, twice-daily milking was resumed for all quarters, and data were collected for an additional 6 wk. Mammary gland biopsies were taken 1 wk before differential milking (wk –1) and after 4 and 8 wk of differential milking. Milk samples were collected weekly throughout the experiment. Once-daily milking resulted in an immediate reduction in milk yield, whereas thrice-daily milking resulted in an increase in milk yield. During differential milking, the daily milk yield of the quarters milked once daily declined by 0.54 kg/wk, on average, but remained constant in the quarters milked thrice daily. Part of the difference in milk yield between the glands pairs persisted after twice-daily milking was reinitiated. In the quarters milked once daily, milk BSA concentration increased, indicating an increase in tight junction leakiness, and zymographic analysis of milk enzymes showed increased activity of several proteases. Reducing the milking frequency also increased mammary cell apoptosis and, surprisingly, mammary cell proliferation. Interestingly, milk concentrations of stanniocalcin-1 and insulin-like growth factor-I and mammary gland expression of several genes were also modulated by milking frequency. For example, expression of insulin-like growth factor I receptor was downregulated during once-daily milking. Last, expression of the short and

long isoforms of the prolactin receptor and of *CSN2* (β -casein) were upregulated during thrice-daily milking. Taken together, these data suggest that milking frequency not only affects mammary gland remodeling and the expression of paracrine factors but also modulates hormone sensitivity.

Key words: differential milking, apoptosis, prolactin, involution

INTRODUCTION

Milk production is a function of the number of mammary epithelial cells and their secretory activity. Capuco et al. (2001) proposed that the decrease in milk production following peak lactation in dairy cows is largely caused by mammary epithelial cell loss. The events leading to this cell loss are poorly understood, however, and a better understanding of this process could be beneficial for dairy producers by suggesting new ways to improve lactation persistency.

It is well known that milking frequency directly influences milk yield (Hale et al., 2003; Nørgaard et al., 2005). Increasing the milking frequency (IMF) in early lactation increases milk yield and has a carryover effect that persists when the period of IMF is finished (Hale et al., 2003; Wall and McFadden, 2007). Conversely, reducing the milking frequency has a negative effect on milk production (Erdman and Varner, 1995; O'Brien et al., 2002). Moreover, these effects are locally regulated, as has been demonstrated in several half-udder studies (Stelwagen and Knight, 1997; Sorensen et al., 2001; Wall and McFadden, 2007). Several hypotheses have been proposed to explain the positive effect of IMF (reviewed recently by Wall and McFadden, 2008), but the exact mechanism remains to be elucidated.

Given that milking frequency modulates lactation persistency, a detailed characterization of the effects of milking frequency could provide relevant information about the regulation of lactation persistency. Accordingly, the objective of this study was to characterize the effects of IMF (thrice-daily milking) and reduced milk-

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ing frequency (once-daily milking) on milk production and composition as well as on mammary cell proliferation, apoptosis, and gene expression.

MATERIALS AND METHODS

Animals and Experimental Procedures

For this study, 10 mid-lactation Holstein cows (7 primiparous and 3 multiparous) averaging 169 ± 9 DIM were used. The cows were housed at Agriculture and Agri-Food Canada's Dairy and Swine Research and Development Centre (Sherbrooke, Quebec) and animal use was conducted in accordance with Canadian Council on Animal Care guidelines. Before the initiation of treatments, all quarters were milked twice daily ($2\times$; wk -3, -2, and -1; pretreatment period). During the following 8 wk (treatment period), the left hindquarters and right forequarters were milked once daily ($1\times$), whereas the right hindquarters and left forequarters were milked thrice daily ($3\times$). After the treatment period, $2\times$ milking was resumed on all quarters and data were collected for an additional 6 wk (wk 9 to 14; posttreatment period). The cows were milked at 0700 and 1900 h during $2\times$ milking. During differential milking, the $3\times$ quarters were milked at 0700, 1300, and 1900 h (intervals of 6, 6, and 12 h), whereas the $1\times$ quarters were milked only at 1900 h. Milk was harvested using individual quarter milking units and production was recorded for each quarter. For wk -2 to 10, the daily milk yield was calculated as the average of daily production recorded on each day of the corresponding week. For wk -3, 11, 12, 13, and 14, the daily milk yield was based on 1 d of measurements per week. Milk samples from each quarter were taken 1 d per week at each milking during that day. For each of those samples, milk lactose, protein, and fat concentrations were determined by near-infrared spectrophotometry, and SCC was determined by an automatic cell counter in a commercial laboratory (Valacta Inc., Ste-Anne-de-Bellevue, Quebec, Canada). In addition, aliquots of the milk samples were defatted by centrifugation (15 min, $1,000 \times g$, 4°C) and frozen. In wk -1, 4, and 8, mammary biopsies from both hindquarters were taken as described by Miller et al. (2006). Biopsies were immediately cut into several pieces that were either snap-frozen in liquid nitrogen (200-mg pieces) or fixed overnight at 4°C in 10% neutral buffered formalin (50-mg pieces). The following day, the fixed tissues were dehydrated with a series of graded ethanol baths, cleared in xylene, and embedded in paraffin. Tissue sections $4\text{ }\mu\text{m}$ thick were then cut and mounted on Superfrost Plus slides (Fisher Scientific Inc., Nepean, Ontario, Canada).

Milk BSA Concentration

The concentration of BSA in milk was measured as an indicator of mammary tight junction permeability. The BSA concentration was determined as described by Tremblay et al. (2009). Forequarter milk samples taken at the 1900 h milking were used for this analysis.

Milk Gelatinase Activity

Because milk proteases may affect mammary gland remodeling, milk gelatinase activities were determined by gelatin zymography. Zymography was performed as described by Tremblay et al. (2009) with the exception that 12% polyacrylamide gels (1.5 mm thick) were used. Forequarter milk samples taken at the 1900 h milking were used for this analysis.

Milk IGF-I and Stanniocalcin-1 Concentrations

Measurement of IGF-I concentrations in defatted milk was performed using a double-antibody RIA as described by Abribat et al. (1993) with the exception that the anti-IGF-I antiserum (#AFP4892898) was obtained from the National Hormone and Peptide Program, and the secondary anti-rabbit antibody was purchased from Linco Research Inc. (St. Charles, MO). Milk stanniocalcin-1 (**STC-1**) concentration was determined by RIA as described by Niu et al. (2000). Forequarter milk samples taken at the 1900 h milking were used for this analysis.

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Assay

Mammary cell apoptosis was measured using terminal deoxynucleotidyl transferase dUTP nick-end labeling (**TUNEL**) with the DeadEnd Colorimetric TUNEL System (Promega Corp., Madison, WI). This assay was performed as recommended by the manufacturer. Cells were then counterstained with a methyl green solution and, following dehydration, mounted with Permount (Fisher Scientific Inc.). Tissue sections were viewed with a Nikon Eclipse 50i microscope (Nikon Corp., Tokyo, Japan) fitted with a QImaging Go-3 digital camera (QImaging Corp., Surrey, British Columbia, Canada). ImageJ software (National Institutes of Health, Bethesda, MD) was used to quantify mammary cell apoptosis in at least 5 nonoverlapping fields for each biopsy.

Ki-67 Immunohistological Detection

Mammary cell proliferation was evaluated by the detection of the Ki-67 antigen using the Histostain-

Table 1. Primers used during real-time PCR

Gene ¹	GenBank accession number	Primer (5' to 3')	Orientation	Amplicon length (bp)
<i>ACTG1</i>	BC147868	CGCACCCTGGCATTGTCAT	Forward	227
		TCCAAGGCGACGTAGCAGAG	Reverse	
<i>BAX</i>	U92569	GACGGCCTCCTCTCCTACTT	Forward	68
		AGCACTCCAGCCACAAAGAT	Reverse	
<i>BCL2</i>	U92434	ACGCCTTTGTGGAGCTGTATG	Forward	69
		CCTTCAGAGACAGCCAGGAGA	Reverse	
<i>CSN2</i>	BC111172	TGAAGGTCCTCATCCTTGCCCT	Forward	75
		CACCAGGTACATTGAGTTCTTCCAG	Reverse	
<i>IGF1</i>	AF404761	CCTGCGCAATGGAATAAAGTC	Forward	83
		AGGCAAAAAGAAATCCAGGGA	Reverse	
<i>IGF1R</i>	X54980	CAACCTCCGGCCTTTTACTCT	Forward	76
		GCTGCAGCCAAGCTTCTCA	Reverse	
<i>IGFBP5</i>	NM001105327	AAAGAGCTACCGCGAGCAAG	Forward	77
		CTGCCATCTCGGAGGTGGT	Reverse	
<i>MYC</i>	AF519455	GACCAGATCCCAGAGTTGGA	Forward	105
		GAGCTTTTGCTGCTCTGCTT	Reverse	
<i>PRLR</i> (short)	NM174155	GCCTTCTCGCCTTGTGTCTATG	Forward	107
		GCAAGTCCTCGCAGTCAGAA	Reverse	
<i>PRLR</i> (long)	NM001039726	TTGATGTTTCATCTGCTGGGAAG	Forward	96
		GCAAGTCCTCGCAGTCAGAA	Reverse	
<i>PTH1H</i>	BC129411	CCTGCTCCCAACACCAAGA	Forward	65
		GTCAGGTATTTGCCCTCATCATC	Reverse	
<i>STC1</i>	NM176669	AAGCTGAAAGTCCTCCTCAGGAA	Forward	114
		TTTGCTGAGGCTGTGATTGC	Reverse	

¹*ACTG1* = actin; *BAX* = Bcl2-associated X protein; *BCL2* = B-cell CLL/lymphoma 2; *CSN2* = β -casein; *IGF1* = insulin-like growth factor 1; *IGF1R* = insulin-like growth factor 1 receptor; *IGFBP5* = insulin-like growth factor binding protein 5; *MYC* = c-Myc; *PRLR* (short) = prolactin receptor, short isoform; *PRLR* (long) = prolactin receptor, long isoform; *PTH1H* = parathyroid hormone-like hormone (PTHrP); *STC1* = stanniocalcin-1.

Plus kit and a mouse anti-Ki-67 antibody (Invitrogen Corp., Burlington, Ontario, Canada). This assay was performed as recommended by the manufacturer with the exception that rehydrated tissues were heated in a microwave in citrate buffer and washes were carried out with PBS 0.05% Tween 20. After detection, cells were counterstained with hematoxylin (Invitrogen Corp.), dehydrated, and mounted with Permount (Fisher Scientific Inc.). Tissue sections were viewed with a Nikon Eclipse 50i microscope fitted with a QImaging Go-3 digital camera. Mammary cell proliferation was quantified by analysis of at least 5 nonoverlapping fields for each biopsy using ImageJ software.

Real-Time PCR

Total RNA was extracted from mammary biopsies using Trizol reagent (Invitrogen Corp.) according to the manufacturer's instructions. The quality of RNA was verified by examination after agarose gel electrophoresis. Treatment with DNase and reverse transcription were performed as described by Delbecchi et al. (2005). The resulting cDNA was diluted 1:50 in water, and 5 μ L was used for amplification and quantification with the Power SYBR Green PCR Master Mix (Applied Biosystems Inc., Foster City, CA). AmpErase UNG (Applied Biosystems Inc.) was added to the mix

(0.1 U per 20- μ L reaction) and primers (Applied Biosystems Inc.) were used at a concentration of 300 nM. Primer sequences are presented in Table 1. The PCR conditions consisted of 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 60 s at 60°C using the 7500 Fast Real-Time PCR System (Applied Biosystems Inc.). A standard curve made with serial dilutions of a pool of reverse transcripts (8 samples from this study) was used to quantify samples. Gene expression was normalized with the values obtained for the *ACTG1* (actin) gene.

Statistical Analysis

Statistical analyses were performed using the MIXED procedure (SAS Institute Inc., Cary, NC) as repeated measurements. The subject was cow (milking frequency) and cow was included as a fixed effect in the model. For the analysis of SCC, data were $\log_{10}$ -transformed before analysis. Effects of treatment relative to the pretreatment period were assessed by analyzing the effect of time within each treatment. Statistical analysis of the real-time PCR results was performed in accordance with the recommendations of User Bulletin #2 for the ABI Prism 7700 Sequence Detection System (December 11, 1997; Applied Biosystems Inc.). Briefly, an ANOVA on $\log_{10}$ of the values normalized individually in relation

Figure 1. Effect of differential milking on daily milk yield. Quarters were milked once (1×; □, dashed line) or thrice (3×; ■, solid line) daily from wk 1 to 8 and twice daily before and after the differential milking period. Quarters milked 3× had higher milk yield than quarters milked 1× during and after differential milking ($P < 0.001$). Data are presented as least squares means $\pm$ SEM of each pair of glands.

to *ACTG1* was performed using the MIXED procedure of SAS with repeated measurements. Protease activities were analyzed with the GLIMMIX procedure of SAS, and there was, therefore, no standard error of the mean. Results were considered statistically significant when $P < 0.05$.

RESULTS

At the beginning of the experiment, all quarters were milked 2× and pairs of glands had similar milk yields ($P > 0.15$; Figure 1). During differential milking, the quarters milked 3× produced more milk ($P < 0.001$) than those milked 1×. Moreover, the milk yield of the 1× gland pair declined ($P < 0.001$) by 0.54 kg/d per week, whereas the yield of the 3× gland pair remained stable ($P > 0.15$) during the treatment period. In the last week of treatment (wk 8), the milk yield of the 1× quarters was only 40% of that of the 3× quarters. A difference persisted even after 2× milking was resumed (wk 9 to 14; $P < 0.001$). During the first posttreatment week (wk 9), the milk production of the 1× quarters was 57% of that of the 3× quarters and in the sixth posttreatment week (wk 14), it was 62%.

Before differential milking began, milk composition was similar in all quarters ($P > 0.15$). Milk lactose concentration (Figure 2A) decreased during 1× milking ($P < 0.001$) and was lower than that of the 3× quarters during ($P < 0.001$) and after differential milking ($P < 0.001$). Lactose yield was lower ($P < 0.001$) in the 1× quarters compared with the 3× quarters, averaging 316 ± 10 and 746 ± 10 g/d for 1× and 3× (total per gland pair), respectively, during differential milking, and 365 ± 20 and 654 ± 20 g/d, respectively, after differential

milking (data not shown). Content in fat (Figure 2B) of the milk from the 1× quarters increased gradually ($P < 0.01$) and was higher than that of the 3× quarters in the wk 6 and 8 of treatment ($P < 0.05$). Protein concentration was unaffected by the treatments, averaging 3.55% in the 1× quarters and 3.56% in the 3× quarters (data not shown). Fat and protein yields were higher in the 3× quarters than in the 1× quarters ($P < 0.001$) during and after differential milking, reflecting the higher milk production of these quarters (data not shown). The milk of the 1× quarters had higher SCC than the milk of the 3× quarters during ($P < 0.05$) and after ($P < 0.01$) differential milking (Figure 3). However, the total number of somatic cells (SCC $\times$ milk production) was unaffected by the treatments ($P > 0.15$; data not shown).

Before differential milking, BSA concentration was slightly higher in the milk from the quarters to be milked 1× ($P < 0.05$; Figure 4) compared with those to be milked 3×. Within the first week of differential milking, milk BSA concentration decreased in the 3× quarters ($P < 0.05$) but increased markedly in the 1× quarters ($P < 0.001$). Consequently, the milk of the 1× quarters had higher BSA content during the treatment period ($P < 0.0001$). During the posttreatment period, milk BSA concentration remained higher in the 1× quarters than in the 3× quarters ($P < 0.001$), but to a lesser extent than during the treatment period.

The presence of several proteases in milk was evaluated by gelatin zymography (Figure 5). Based on the apparent molecular weight at which the gelatinolytic activities were detected and on the results obtained by Raulo et al. (2002), we identified 3 matrix metalloproteinases (MMP), namely MMP-1, MMP-2, and MMP-9, as being abundant in our samples. Before differential milking, no differences in the activities of these proteases were observed ($P > 0.15$). However, 1× milking caused an increase in the activity of MMP-1 and MMP-9 ($P < 0.001$ and $P < 0.01$, respectively). Therefore, their activity was higher in the milk from the 1× quarters than in that of the 3× quarters during ($P < 0.01$) and after ($P < 0.01$) the treatment period. Moreover, milk MMP-2 activity was higher in 1× quarters than in the 3× quarters in wk 6 ($P < 0.05$) and 7 ($P < 0.01$), whereas a trend was apparent in wk 8 ($P = 0.07$).

Once-daily milking caused an increase in milk concentration of both IGF-I and STC-1 (Figure 6). This resulted in higher levels of both these hormones in the 1× quarters compared with the 3× quarters (IGF-I, $P < 0.0001$; STC-1, $P < 0.01$), a difference that persisted beyond the treatment period (IGF-I, $P < 0.001$; STC-1, $P < 0.01$). Before differential milking, IGF-I concentration was already higher in the milk from the quarters to be milked 1× than in the milk from those to be milked

Figure 2. Effect of differential milking on milk lactose (A) and fat (B) concentrations. Quarters were milked once (1×; □, dashed line) or thrice (3×; ■, solid line) daily from wk 1 to 8 and twice daily before and after the differential milking period. Milk lactose concentration was lower in the 1× quarters than in the 3× quarters during and after differential milking ($P < 0.001$). In wk 6 and 8 of treatment, fat content was higher in the milk from the 1× quarters than that of the milk from the 3× quarters ($P < 0.05$). Data are presented as least squares means $\pm$ SEM.

3× ($P < 0.05$), whereas milk STC-1 content was similar in all quarters ($P > 0.15$).

Mammary cell apoptosis was measured by TUNEL assay in tissues collected in wk -1, 4, and 8 (Figure 7A). Apoptosis rate was similar in both groups before the treatments ($P > 0.15$) and milking 1× increased the apoptosis rate ($P < 0.05$). In wk 4, it was 2.2 times greater in the 1× quarters than in the 3× quarters ($P < 0.05$). Apoptosis rate was similar in 1× and 3× quarters in wk 8 ($P > 0.15$). Mammary cell proliferation was evaluated by immunohistological detection of the Ki-67 antigen (Figure 7B). Proliferation rate was similar in both groups before treatment. Once-daily milking increased proliferation in the 1× glands ($P < 0.001$) and was 2.4 and 2.8 times higher in the 1× quarters than in the 3× quarters in wk 4 ($P < 0.05$) and wk 8 ($P < 0.01$), respectively.

The mammary gland expression of several genes was evaluated by real-time PCR from tissues taken by biopsy in wk -1, 4, and 8 (Table 2). The expression of *CSN2* (β -casein) decreased with time in the 1× quarters (time $\times$ treatment; $P < 0.001$) but was relatively stable in the 3× quarters (time $\times$ treatment; $P > 0.15$). Thus, *CSN2* mRNA was more abundant in the 3× quarters than in the 1× quarters in wk 4 and 8. The expression of both *IGF1* and *IGF1R* (insulin-like growth factor 1 receptor) was higher in the 3× quarters than in the 1× quarters in wk 4 of treatment, whereas no differences in *IGFBP5* (insulin-like growth factor binding protein 5) expression were observed. In wk 4 and 8, the expression of both *PRLR* (prolactin receptor) isoforms and of *STC1* was significantly higher in the 3× quarters than in the 1× quarters. The expression of *PTH1H* (parathyroid hormone-like hormone) increased with time in the 3× quarters (time $\times$ treatment; $P < 0.01$), whereas it decreased in the 1× quarters (time $\times$ treatment; $P < 0.05$). Also, the expression of the *MYC* gene (c-Myc) increased with time in the 1× quarters (time $\times$ treatment; $P < 0.05$) but was relatively stable in the 3× quarters (time $\times$ treatment; $P > 0.15$). Thus, the 1× quarters expressed more *MYC* than the 3× quarters in wk 8. Expression of *BAX* (Bcl2-associated X protein) was similar in both glands, whereas expression of *BCL2* (B-cell CLL/lymphoma 2) was higher in the 1× quarters at wk 8. Consequently, the *BAX:BCL2* mRNA ratio was lower in the 1× quarters than in the 3× quarters in wk 8 of treatment.

DISCUSSION

In the present experiment, mid-lactation cows were milked at different frequencies according to a half-udder model to characterize the effects of milking frequency on the mammary gland and on the production and composition of milk. This study was also designed to identify factors that may be implicated in the modulation of lactation persistency.

In agreement with previous studies, 1× milking reduced milk yield (O'Brien et al., 2002), whereas 3× milking increased it (Hale et al., 2003). After 8 wk of treatment, 2× milking was resumed and an important, although partial, difference in yield remained between the gland pairs that had been milked at different frequencies. Interestingly, the milk yield of the 3× gland pairs was numerically higher (0.8 kg/d) during the first week of posttreatment than the average production measured during the pretreatment period, even though it was more than 2 mo later. Although in agreement with the work of Elliott (1961), this positive effect of 3× milking on persistency is in discrepancy with a recent review by Wall and McFadden (2008), which stated that

Figure 3. Effect of differential milking on milk SCC (cells/mL) in quarters milked once (1×; □, dashed line) or thrice (3×; ■, solid line) daily from wk 1 to 8 and twice daily before and after the differential milking period. The 1× quarters had higher milk SCC than the 3× quarters during ($P < 0.05$) and after ($P < 0.01$) differential milking. Data were $\log_{10}$ -transformed before statistical analysis and are presented as the antilog values of the calculated means.

the positive carryover effect of IMF is limited to early lactation. It therefore seems that at any stage of lactation differential milking may have 2 important effects on lactation: an immediate but sustained modulation of milk production and a longer term effect on lactation persistency. It is important to note that reduced milking frequency in one gland pair could have increased the nutrients available for the other half. This could have had a positive effect on milk production. However, the carryover effect caused by 3× milking strongly suggests that nutrient availability is not solely responsible for the positive effects of IMF.

Once-daily milking had numerous other effects on the mammary gland. Milk BSA concentration increased rapidly when milking frequency changed, whereas lactose concentration decreased. Consequently, it seems that 1× milking led to an increase in the permeability of the mammary epithelium. This was probably caused by an increase in the leakiness of the tight junctions. As discussed by Stelwagen (2001), this permeability change could be responsible for the rapid decrease in milk yield observed upon initiation of 1× milking. Although BSA concentrations declined when milking frequency increased from 1× to 2×, it remained higher than in the milk from the half gland previously milked 3×. A carryover effect was also observed by Stelwagen and Lacy-Hulbert (1996). Similarly, SCC remained elevated in milk from these quarters. Diapedesis of immune cells will disturb epithelial cell integrity and probably provide opportunities for BSA to diffuse into milk. Accordingly, mammary inflammation causes a sharp increase in milk concentration of BSA (Bouchard et al., 1999).

Once-daily milking increased mammary cell apoptosis and, surprisingly, also mammary cell proliferation. Mammary gland expression of the *MYC* gene was also upregulated, and given that the product of this gene can promote mammary cell proliferation, the *MYC* gene product may have played an important role in the observed increase in cell proliferation. To our knowledge, this is the first report indicating that an increased rate of mammary cell proliferation is induced by reduced milking frequency. However, 1× milking seems to have led to a net loss of mammary tissue. Indeed, visual inspection of the quarters after milking revealed an obvious reduction in the size of the 1× quarters. During the entire posttreatment period (after 2× milking had been resumed), the quarters that had previously been milked 1× were still smaller than those that had been milked 3×. Thus, mammary cell loss is probably responsible for the negative effect of 1× milking on lactation persistency. Reduced mammary secretory activity could also have contributed to this effect, given that *CSN2* expression was decreased during the 1× milking. However, this reduced gene expression could also be the consequence of the loss of secretory cells.

Milk gelatinase activity of MMP also increased during 1× milking. Substrates of these proteases include several extracellular matrix components present in the mammary gland (Chakraborti et al., 2003). Therefore, the increase in MMP activities was probably involved in the remodeling of the mammary gland. It is important to mention that inhibition of the MMP by inhibitors is lost during zymography (Snoek-van Beurden and Von den Hoff, 2005); consequently, the measured MMP activities could be different from the actual in

Figure 4. Effect of differential milking on milk BSA concentration in quarters milked once (1×; □, dashed line) or thrice (3×; ■, solid line) daily from wk 1 to 8 and twice daily before and after the differential milking period. Milk BSA concentration was higher in the 1× quarters than in the 3× quarters during and after differential milking ($P < 0.001$), and to a lesser extent, in the pretreatment period ($P < 0.05$). Data are presented as least squares means $\pm$ SEM.

Figure 5. Effect of differential milking on milk matrix metalloproteinase (MMP) activities. Activity of A) MMP-1, B) MMP-2, and C) MMP-9 in milk obtained from quarters milked once (1 $\times$; $\square$, dashed line) or thrice (3 $\times$; $\blacksquare$, solid line) daily from wk 1 to 8 and twice daily before and after the differential milking period. The activity of MMP-1 and MMP-9 was higher in the 1 $\times$ milk than in the 3 $\times$ milk during and after differential milking ($P < 0.01$). Differences in milk MMP-2 activities were observed in wk 6 ($P < 0.05$) and 7 ($P < 0.01$), whereas a trend was apparent in wk 8 ($P = 0.07$). Data are presented as least squares means.

vivo activities. Interestingly, milk MMP activities, BSA concentration (Tremblay et al., 2009), and mammary cell proliferation (Capuco et al., 1997) and apoptosis (Nørgaard et al., 2008) are similarly increased during the mammary gland remodeling that occurs during the dry period. Given that these parameters were also increased by 1 $\times$ milking in this study, it seems that a similar mammary gland remodeling was induced by 1 $\times$ milking.

Another interesting event that took place in the 1 $\times$ quarters was a decrease in *IGF1R* expression. Although the expression of *IGF1* and *IGFBP5* genes or changes in milk IGF-I concentration cannot explain the effect of 1 $\times$ milking on production and persistency, the expression of this IGF-I receptor could. It has been demonstrated that IGF-I protects various cell types against multiple apoptotic stimuli, including tumor necrosis factor- α (TNF- α) and Fas ligand (FasL), mainly by activating PI3K/AKT and ERK1/2 (Kurmashева and Houghton, 2006). Interestingly, the mammary gland expression of both TNF- α and FasL is upregulated during mouse mammary gland involution induced by milk stasis (Watson, 2006). Similarly, it appears that c-Myc may induce apoptosis, which can be inhibited by IGF-I under certain conditions (Harrington et al., 1994; Hueber et al., 1997). Although not evaluated in this study, the increased expression of *MYC* and possibly of *TNF* and *FASLG* (genes encoding TNF- α and FasL, respectively), coupled with reduced IGF-I responsiveness, may have played an important role in the loss of mammary tissue in the 1 $\times$ quarters. Therefore, the decreased expression of *IGF1R* could be implicated in the reduced persistency of the 1 $\times$ quarters.

Milking 3 $\times$ had more limited effects on several parameters than did milking 1 $\times$. For example, milk MMP activities, lactose concentration, and mammary cell proliferation and apoptosis were unaffected by 3 $\times$ milking. However, unlike 1 $\times$ milking, 3 $\times$ milking decreased milk BSA concentration, although BSA concentration returned to pretreatment values when 2 $\times$ milking was reinitiated. This result suggests that frequent milking reduces tight junction leakiness and could have had a positive effect on milk yield. Accordingly, Sorensen et al. (2001) observed better epithelial integrity in half glands milked 3 $\times$ compared with half glands milked 2 $\times$.

Given that milk production is a function of the number of mammary epithelial cells and their secretory activity, one or both parameters must have been positively affected by IMF. The increased secretory activity likely contributed to the rapid increase in milk production during 3 $\times$ milking, given that *CSN2* expression was upregulated by this treatment. Despite the fact that we have not been able to show a clear effect

Figure 6. Effect of differential milking on milk IGF-I (panel A) and stanniocalcin-1 (STC-1, panel B) concentrations in milk obtained from quarters milked once (1×; □, dashed line) or thrice (3×; ■, solid line) daily from wk 1 to 8 and twice daily before and after the differential milking period. Milk IGF-I and STC-1 concentrations were higher in the 1× quarters than in the 3× quarters during (IGF-I, $P < 0.0001$; STC-1, $P < 0.01$) and after differential milking (IGF-I, $P < 0.001$; STC-1, $P < 0.01$). Milk IGF-I concentration was already higher in those quarters before treatment ($P < 0.05$). Data are presented as least squares means $\pm$ SEM.

of 3× milking on mammary proliferation and apoptosis, a reduction of the net loss of secretory cells during the 8 wk of treatment probably contributed to the positive effect of IMF on lactation persistency. However, with only the present data we cannot rule out the possibility that increased mammary cell secretory activity may also have contributed to this effect.

Although the expression of both *PRLR* isoforms was higher in the 3× quarters, the role of prolactin (**PRL**) in lactating dairy ruminants is a matter of debate. Plaut et al. (1987) reported that the administration of pharmacological doses of PRL did not affect milk yield. In older studies, short-term inhibition of PRL secretion by CB154 resulted in little or no effect on milk yield (Karg and Schams, 1974). Nevertheless, Lacasse et al. (2008) recently found that treating cows for a longer period with quinagolide, a powerful PRL-release inhibi-

Figure 7. Effect of differential milking on apoptosis (A; terminal deoxynucleotidyl transferase dUTP nick-end labeling, TUNEL) and proliferation (B; Ki-67 labeling) rates of mammary cells obtained from quarters milked once (1×; gray bars) or thrice (3×; black bars) daily during the differential milking period and twice daily during the pre-treatment period, with biopsies taken in wk -1, 4, and 8. Data are presented as the percentage of labeled cells compared with the total number of cells. Apoptosis was higher in the 1× quarters than in the 3× quarters in wk 4 ($P < 0.05$), whereas proliferation was higher in wk 4 ($P < 0.05$) and wk 8 ($P < 0.01$). Data are presented as least squares means $\pm$ SEM.

tor, reduces milk production. Those authors also found that, when combined with differential milking (half of the gland milked 2× and the other half milked 1×), quinagolide loses its inhibitory effect in the 1× glands. This result suggests there is a link between the effect of PRL and milking frequency, as supported by the present data. Moreover, if PRL acts as a galactopoietic hormone in cows, higher responsiveness to PRL caused by upregulated *PRLR* expression could have resulted in increased mammary cell secretory activity and lactation persistency. Therefore, the upregulation of *PRLR* isoforms could be critical for the positive effects of IMF.

In accordance with a previous study (Miller et al., 2006), we observed an increase in milk STC-1 concentration as lactation progressed. Moreover, this increase

Table 2. Gene expression in the mammary glands of lactating cows milked at different frequencies¹

Gene ²	Pretreatment			Week 4			Week 8		
	Left 2×	Right 2×	SEM	Left 1×	Right 3×	SEM	Left 1×	Right 3×	SEM
<i>BAX</i>	1.019	0.959	0.323	0.949	1.273	0.308	1.286	1.174	0.387
<i>BCL2</i>	1.044	0.917	0.162	1.487	1.069	0.344	2.270	1.112*	0.487
<i>CSN2</i>	1.239	1.199	0.092	0.834	1.603***	0.153	0.483	1.286***	0.193
<i>IGF1</i>	0.820	0.833	0.108	0.825	1.265*	0.195	1.450	1.020	0.286
<i>IGF1R</i>	0.594	0.556	0.111	0.292	0.530*	0.121	0.308	0.505†	0.130
<i>IGFBP5</i>	1.632	1.786	0.262	1.707	1.451	0.289	1.349	1.331	0.261
<i>MYC</i>	0.881	0.899	0.078	1.424	1.053	0.338	1.929	0.947**	0.350
<i>PRLR</i> (short)	0.897	0.766*	0.078	0.704	0.943**	0.091	0.646	0.954**	0.111
<i>PRLR</i> (long)	0.779	0.690	0.061	0.724	0.893*	0.064	0.699	0.965*	0.106
<i>PTH1H</i>	1.203	1.131	0.125	0.983	1.780***	0.179	0.485	1.309***	0.191
<i>STC1</i>	1.219	1.150	0.108	0.902	1.686***	0.136	1.158	1.995**	0.232
<i>BAX:BCL2</i>	0.873	1.064†	0.136	0.871	1.053	0.175	0.515	1.006***	0.141

¹Mammary biopsies were taken on hindquarters in the indicated week (pretreatment corresponds to wk -1). Quarters were milked twice daily (2×) during the pretreatment period and either once (1×) or thrice daily (3×) during the differential milking period (wk 4 and 8). All values were normalized with the *ACTG1* cDNA and log₁₀ transformed.

²*ACTG1* = actin; *BAX* = Bcl2-associated X protein; *BCL2* = B-cell CLL/lymphoma 2; *CSN2* = β-casein; *IGF1* = insulin-like growth factor 1; *IGF1R* = insulin-like growth factor 1 receptor; *IGFBP5* = insulin-like growth factor binding protein 5; *MYC* = c-Myc; *PRLR* (short) = prolactin receptor, short isoform; *PRLR* (long) = prolactin receptor, long isoform; *PTH1H* = parathyroid hormone-like hormone (PTHrP); *STC1* = stanniocalcin-1; *BAX:BCL2* = *BAX* to *BCL2* mRNA expression ratio.

†*P* < 0.10; **P* < 0.05; ***P* < 0.01; ****P* < 0.001 indicate differences between left and right hindquarters.

was higher in the 1× milk than in the 3× milk. Previous studies also showed that prolonged milk stasis increases the concentration of this calcium-regulating hormone in milk (Tremblay et al., 2009). Similarly, in response to injections of 17β-estradiol (Delbecchi et al., 2005), which led to important reductions in milk production, milk concentration of STC-1 was increased. Consequently, the elevated STC-1 concentrations in 1× milk could be related to the decrease in milk yield, mammary gland remodeling, or both. Thus, we suspect that STC-1 could be a negative regulator of lactation. Mammary *STC1* expression levels did not correlate with its concentration in the milk, suggesting that blood-borne STC-1 is present in milk. Interestingly, milking frequency also modulated the expression of another calcium-regulating hormone. Although 1× milking decreased mammary gland expression of the gene encoding the parathyroid hormone-related protein (**PTHrP**; *PTH1H* gene), the expression of that gene was increased during 3× milking. Thompson et al. (1994) reported a similar modulation of PTHrP in goats milked unilaterally 1× and 4×. Several functions have been proposed for this hormone in the mammary gland (Philbrick et al., 1996) but the observed modulation of its expression by milking frequency was likely a response to a change in the calcium requirement for milk production. Interestingly, STC-1 and PTHrP could play opposing roles in this process, given that it was recently shown that STC-1 can inhibit some of the effects of PTHrP in fish (Wever et al., 2007). Stelwagen and Callaghan (2003) reported that PTHrP plays a role

in the maintenance of the mammary tight junctions. Interestingly, a negative relationship between *PTH1H* gene expression and tight junction permeability was observed in the present study.

Because the *BAX:BCL2* expression ratio is often used as an indicator of cell susceptibility to apoptosis (Perlman et al., 1999; Nørgaard et al., 2008), the expression levels of both genes were evaluated. Interestingly, their ratios did not correlate with the apoptosis rate measured by TUNEL assay. Given that the TUNEL assay is a more direct measurement of apoptosis, it seems that the *BAX:BCL2* ratio gave an inaccurate indication of cell susceptibility to apoptosis. Considering the large number of Bcl-2 family members regulating apoptosis, this is not a surprising result. The *BAX:BCL2* ratio should therefore be interpreted with caution.

This study has shown that milking frequency modulates both milk yield and lactation persistency in mid-lactation cows. Interestingly, milking 1× seems to increase mammary remodeling. Our results also suggest that milking frequency may affect mammary gland responsiveness to endocrine factors such as PRL and IGF-I. However, information is still lacking on the local mediators that link the frequency of milk removal and the events observed at the cellular level. The involvement of factors such as STC-1 and the implications of mammary gland permeability are possible but not yet proven. In conclusion, the results of this study suggest that the management of milking frequency has the potential to be beneficial not only during early lactation but also later in lactation.

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