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Mechanism underlying the modulation of milk production by incomplete milking

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ABSTRACT

Mammary gland secretory activity is modulated by systemic and local factors; however, the relationship between these factors is unknown. The aim of this study was to determine how a local factor, such as incomplete milking, affects mammary epithelial cell activity, number, and responsiveness to blood prolactin (PRL). Eight cows in mid-lactation were differentially milked (i.e., their right quarters were milked incompletely at approximately 70%, and their left quarters were milked completely, twice daily for 4 wk). Throughout the experiment, milk yield was measured at the quarter level. Milk samples were collected from each quarter once a week to assess the milk components, and epithelial cell concentrations, as well as to isolate milk fat globule RNA. In the weeks before and after the experiment, mammary gland functional capacity was evaluated by measuring the volume of milk harvested after complete filling of the gland. At the end of the last experimental week, mammary gland biopsies were performed on each rear quarter. The milk production of quarters milked completely remained stable during the treatment period, whereas, as expected, the milk production of quarters milked incompletely was only 53% of completely milked quarters at the end of the period. Accordingly, the expression of genes related to milk synthesis (*CSN2*, *LALBA*, and *ACACA*) in milk fat was lower in the quarters that were milked incompletely. Incomplete milking decreased the milk lactose content, indicating a loss of integrity of tight junctions. The total yield of epithelial cells in milk was not affected, but their concentration in milk, the *BAX:BCL2* gene expression ratio, and the loss of mammary functional capacity were greater in the quarters milked incompletely, suggesting an acceleration of involution in those quarters. The expression of the short isoform of the PRL receptor gene (*PRLR*) tended to be lower, and the expression of *STAT5A* and *STAT5B* tended to decline in the quarters milked incompletely. In mammary gland biopsy samples, the number of both short and long isoforms of the PRLR were not affected, nor were the amount and activation of STAT3 and STAT5. However, the ratio of PRLR short isoform to PRLR long isoform was lower in the quarters milked incompletely. The decrease in milk yield induced by incomplete milking is rapid and associated with a decrease in mammary epithelial cell activity and a decrease in the number of secretory epithelial cells. The results of this experiment provide only limited support for the hypothesis that modulation of the mammary gland's responsiveness to PRL is part of the mechanism by which local factors, such as incomplete milking, modulate milk synthesis.
Key words: mammary gland, apoptosis, involution, prolactin

INTRODUCTION

A certain amount of milk remains in the mammary gland even when the milking machine is removed after the milk flow has stopped completely. The amount of milk left in the gland increases with early termination of milking or an inefficient milking routine. An increase in the milk not removed from the mammary gland at milking is linked to a reduction of milk production (Woodward et al., 1936), whereas a reduction of residual milk through oxytocin injection increases production (Lollivier and Marnet, 2005). The inhibitory effect of incomplete milking increases with its intensity and duration (Schmidt et al., 1964; Wheelock et al., 1965), and when this method is applied for several days, the effect persists even after complete milking is resumed (Schmidt et al., 1964). The effect is controlled locally because, in a split-udder design, the effect is limited to the incompletely milked quarters (Elliott, 1961; Penry et al., 2017).

Although the effect of incomplete milking has been known for a long time, the mechanism by which it

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affects milk production is still unknown. Conversely, the mechanism by which milking frequency alters milk production has been the object of several studies. Differential milking experiments, in which different milking frequencies are applied according to a split-udder design, have shown that changes in milk production are locally regulated (Bernier-Dodier et al., 2010; Alex et al., 2015). Recently, Toledo et al. (2020) have shown that milking frequency affects both the mammary epithelial cells' secretory activity and their number, in modulating milk production.

Prolactin (**PRL**), as its name implies, is an important hormone for the control of lactation, and its suppression inhibits milk production in dairy cows (Lacasse et al., 2011). Furthermore, apoptosis of mammary epithelial cells was greater in the mammary glands of cows in which PRL secretion was inhibited compared with those in which PRL was not inhibited, suggesting that PRL plays a role in the survival of mammary cells (Boutinaud et al., 2012). For a comprehensive review of the galactopoietic role of PRL in ruminants, readers are invited to consult the review paper by Lacasse et al. (2016). Increasing milking frequency increases the number (McKinnon et al., 1988) and gene expression (Bernier-Dodier et al., 2010; Toledo et al., 2020) of PRL receptors in the mammary gland, providing another potential link between systemic and local regulation of the response in the mammary gland. In addition, the total amount of phosphorylated STAT5 protein, which is believed to be an intracellular mediator of PRL signal (Teglund et al., 1998), increases with milking frequency (Toledo et al., 2020). Because there are several similarities between reduced milking frequency and incomplete milking, we hypothesized that both affect lactation persistency by the same mechanism. The objective of this study was to determine how incomplete milking affects mammary epithelial cell activity, cell number, and cell responsiveness to blood PRL.

MATERIALS AND METHODS

Animals and Experimental Design

The present study was conducted in accordance with the rules and guidelines of the Canadian Council on Animal Care (1993) and approved by the Sherbrooke Research and Development Center's animal care committee (#550). Eight multiparous and gestating mid-lactation (DIM 174 ± 45) Holstein cows with SCC below 240,000/mL from the herd at the Sherbrooke Research and Development Center (Sherbrooke, QC, Canada) were included in the experiment. During the first 2 experimental weeks (wk −2 and −1), the cows were completely milked twice daily at 12-h intervals (pretreatment period). During wk −1, quarter milking was initiated, and the time needed to empty the right quarters was determined for each cow. During wk 1 to 4 (treatment period), the cows were differentially milked at 12-h intervals; the left quarters were completely milked (**COMP**), whereas the right quarters were incompletely milked (70%, **INC**). On wk −1, we determined, for each cow and each quarter (front and rear), the time at which milking needed to be stopped to leave 30% of the total yield of the right quarters in the gland based on the times and testing done in wk −1. These times were re-evaluated and adjusted at the end of wk 2. Milk yield was recorded at each milking. Cows were fed the same diet throughout the experiment.

Milk Composition

Milk samples were collected weekly, between wk −1 and 4, at the morning milking for each quarter. The milk components of whole milk from each quarter were analyzed for lactose, protein, fat, and SCC concentrations in a commercial laboratory (Lactanet).

Mammary Functional Capacity

In wk −1 and 6 (12 d after the cessation of treatment), mammary functional capacity was determined by measuring the volume of milk harvested after complete filling of the gland (26-h milking interval) and a final intramuscular injection of oxytocin (20 IU) was administered to collect residual milk, as described by Toledo et al. (2020).

Milk Samples: RNA Extraction

Aseptic quarter milk samples were manually collected weekly from wk −1 to 4 in sterile 50-mL tubes and kept on ice after proper disinfection of the teats. The samples were then centrifuged to extract milk fat which was then put in TRIzol LS Reagent (Life Technologies) as described by Toledo et al. (2020) and stored at −80°C until further RNA extraction. Before extraction, the samples were centrifuged and the middle Trizol phase collected for milk fat globule RNA extraction as described by Tong et al. (2018). The RNA was then extracted using TRIzol LS and the PureLink RNA Mini Kit, which includes the optional on-column DNase treatment (Invitrogen), according to the manufacturer's instructions. After extraction, RNA amounts were quantified using a BioDrop μLITE spectrophotometer (BioDrop Ltd.), and the quality of the RNA

Table 1. Primers used during real-time PCR (F = forward; R = reverse)

Gene[1]	GenBank number	Primer (5′–3′)	Amplicon size (bp)
ACTB	NM_173979.3	F: TGGCACCCAGCACAATGA R: CCTGCTTGCTGATCCACATCT	123
UXT	NM_001037471.2	F: TGGCAGAAGCTCTCAAGTTCATT R: CATGTGGATATGGGCCTTGAT	104
GAPDH	NM_001034034.2	F: GCGAGATCCTGCCAACATCAAG R: TCAAGTGAGCCCCRGCCTTC	101
RPS24	NM_001025339.2	F: CTCTTTTCCTCCCTGGCTGC R: TCCGCTGAAGCAGTCGGTTG	100
BAX	NM_173894.1	F: GGGTTGTCGCCCTTTTCTACTT R: CGCTCTCGAAGGAAGTCCAATG	115
BCL2	NM_001166486.1	F: ACAGCATCGCCCTGTGGATG R: GTGCCTTCAGAGACAGCCAG	147
SOCS3	NM_174466.2	F: AGAAGATCCCTCTGGTGTTGAGC R: GTGACTTTCTCGTAGGAGTCCAGG	109
CSN2	XM_010806178.1	F: AGCAAACAGAGGATGAACTCCAGG R: AGGGATGTTTTGTGGGAGGCTG	110
LALBA	NM_174378.2	F: CTCTGCTCCTGGTAGGCATC R: ACAGACCCATTCAGGCAAAC	125
ACACA	NM_174224.2	F: CATGTATGGACACCAGTTCTGCAT R: GTTTGGTAGGACATCAAAAATCGA	82
$PRLR_{long}$	NM_001039726.2	F: TCATCTGCTGGAGAAGGGCAA R: CTGGTCCTCACAGTCATCTACCTC	127
$PRLR_{short}$	NM_174155.3	F: TATCACAGCCTTCTCGCCTTG R: CTGGTCCTCACAGTCATCTACCTC	152
STAT5A	NM_001012673	F: GTGCCCCCAGCCTCACTATAAC R: GTAAAGAGACCAGTGGGCGG	174
STAT5B	NM_174617	F: GTTTCGTGAACAAGCAACAGGC R: GGCATCAGATTCCAAAACATTCTTTCC	148

[1]*ACTB* = β-actin, *UXT* = ubiquitously expressed transcript; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *RPS24* = ribosomal protein S24; *BAX* = BCL2-associated X protein; *BCL2* = B-cell CLL/lymphoma 2; *SOCS3* = suppressors of cytokine signaling 3; *CSN2* = β-casein; *LALBA* = α-lactalbumin; *ACACA* = acetyl-coenzyme A (CoA) carboxylase α; $PRLR_{long}$ = prolactin receptor, long isoform; $PRLR_{short}$ = prolactin receptor, short isoform; *STAT5A* = signal transducer and activator of transcription 5A; *STAT5B* = signal transducer and activator of transcription 5B.

was assessed using a Bioanalyzer (2100 Bioanalyzer Instrument, Agilent). Last, all samples were frozen and stored at −80°C for further gene expression analysis.

Mammary Biopsies

At the end of wk 4, mammary gland biopsies were obtained from the rear quarters. Mammary tissue was collected, rinsed in sterile saline solution to remove traces of blood, cut in small pieces and then immediately frozen in liquid nitrogen and stored at −80°C for further analysis as previously described by Tong et al. (2018).

Quantitative Reverse-Transcription PCR

The abundance of genes related to PRL signaling [long and short PRL receptor ($PRLR_{long}$ and $PRLR_{short}$), *SOCS3*, *STAT5A*, and *STAT5B*], mammary activity (*LALBA*, *CSN2*, and *ACACA*), and apoptosis (*BCL2* and *BAX*) in RNA was determined by a 2-step quantitative reverse-transcription PCR. Gene-specific primer sets (Table 1) for all genes were either designed using the Ensembl gene browser (Yates et al., 2016) in accordance with the primer design of Brosseau et al. (2010), or were described in previous studies (Thompson et al., 2011; Dudemaine et al., 2014) and synthesized by Integrated DNA Technologies Inc. Reverse-transcription reaction for each sample was conducted in 750 ng of total RNA with qScript cDNA Supermix (Quantabio), in accordance with the manufacturer's instructions. Quantitative triplicate PCR reactions were completed for each sample and were run in parallel with a relative standard curve formed by pools of all samples. Briefly, each PCR reaction was executed in a 10-μL volume with PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) and 300–500 n*M* of gene-specific primer in a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific).

The *ACTB*, *GAPDH*, *UXT*, and *RPS24* genes were quantified to select reference genes. The NormFinder (Andersen et al., 2004) program was used to analyze the expression stability of candidate reference genes. The genes with the most stable expression were *GAPDH* and *RPS24* and, therefore, were used as the reference genes in this study. The abundance of the target genes

was determined as the relative quantity ratio by dividing the relative quantity of the targets by the geometric mean of the relative quantity of the reference genes.

Determination of Prolactin Receptors and of STAT Protein and Activation Levels (Western Blot)

Western blot analysis was carried out as described by Tong et al. (2018) to determine the abundance of the short and the long isoforms of PRLR in mammary tissue. The abundance or activation of STAT proteins (STAT5 A and B, and STAT3) was determined using a commercial ELISA kit (Abcam).

Determination of Epithelial Cell Concentrations in Milk

In wk −1, 1, 2, 3, and 4, milk samples from whole milking were collected at morning milking from each quarter. The samples were collected in 50-mL sterile tubes and kept at room temperature. Then, 15 mL of each sample was diluted at a 1:3 ratio with PBS (Wisent Inc.) and centrifuged at 1,000 × *g* for 15 min at room temperature. The milk was then skimmed and the supernatant discarded. The cell pellet was suspended in 15 mL of PBS containing 1% BSA (Sigma-Aldrich Canada Co.) and 2% normal goat serum (normal goat serum sterile FILT, Cedarlane) and centrifuged at 500 × *g* for 10 min at room temperature. This was repeated in 20 mL of PBS with 1% BSA and 2% normal goat serum. The supernatant was again discarded, and the cell pellet was resuspended in exactly 1.5 mL of PBS with BSA and normal goat serum (1% and 2%). Next, 100 μL of each sample was then distributed in duplicate in a U-bottom 96 wells plate (Falcon 96-well assay plates, VWR). A pool of each sample collected and treated on the day of the experiment was also added and used as the control wells for the antibodies. Each sample was then incubated with an antibody mix containing anti-CD45 (mouse anti-sheep CD45 Biotin, Bio-Rad and brilliant violet 605 Streptavidin, Cedarlane), anti-CD49f (PE/Cy7 anti-human/mouse CD49f, Cedarlane) and anti-epithelial cell cytoskeletal proteins (Alexa Fluor 488 mouse anti-human vimentin, clone RV 202, BD Bioscience Canada) as well as a viability cell marker (Ghost Dye Violet 510, Cedarlane). Last, 10 μL of counting beads (0.52 E05/50 μL; CountBright absolute counting beads, Thermo Fisher Scientific) were added to determine the concentration of epithelial cells in each sample after reading in a BD FACS Canto II flow cytometer. After the reading, different zones were identified to isolate the cells of interest. The counting beads were gated using a forward-versus-linear-side scatter plot, and 5,200 bead events were acquired. Additional gates were selected for the cells marked by the anti-CD45 antibody, for those marked by CD49f and vimentin, and last, for those marked by the viability cell markers. The concentration of epithelial cells per microliter of milk was then determined based on the number of beads, the volume of milk used for the experiment, and the number of events in the gates of interest, was calculated as (number of events of vimentin and CD49f positives and CD45 negatives)/(number of bead events × 500/number of beads in 10 μL).

Statistical Analysis

Data were analyzed by ANOVA using the MIXED procedure of SAS (version 9.0, SAS Institute Inc.). Time was used as a repeated effect. The experimental unit was the half-udder, and the observational unit was the quarter; therefore, statistical analysis were carried out on quarter data, but are reported at for the half-udder. Means data from before the onset of treatments were, when available and significant, used as a covariate. Differences were considered statistically significant when $P < 0.05$ and were considered a trend when $P < 0.10$.

RESULTS

Milk Yields and Milk Composition

Before the initiation of treatments, we observed no difference in milk production between left and right half-udders ($P = 0.35$; Supplemental Figure S1; https://data.mendeley.com/datasets/txvr8jzy4k/1; Lacasse, 2022). During the treatment period, milk production remained stable in the half gland milked completely (Figure 1). As expected, milk production in the INC half gland was lower ($P < 0.001$), representing about 66% of milk production in the COMP half gland at the start of the treatment period, and 53% at the end. Adjustments made to milking time for the INC half gland at the end of wk 2 (d 14) explains the decline observed after d 14. Before the onset of treatments, milk fat ($P = 0.84$), protein ($P = 0.80$), and lactose ($P = 0.18$) did not differ between the half glands. During the treatment period, the milk contents of fat ($P = 0.70$) and protein ($P = 0.48$) did not differ between the quarters milked completely and those milked incompletely (Table 2). Milk lactose content was lower ($P < 0.001$) in the quarters milked incompletely. This effect tended to increase during the treatment period (treat-

Table 2. Composition of milk from half glands milked completely (n = 8) or incompletely (n = 8) for 4 wk[1]

Variable	Complete milking	Incomplete milking	SEM	*P*-value
Milk yield (kg/d)	23.12	13.98	0.16	<0.001
Fat (%)	3.62	3.57	0.10	0.70
Fat yield (g/d)	411.04	246.7	11.08	<0.001
Protein (%)	3.26	3.28	0.02	0.48
Protein yield (g/d)	368.81	223.24	7.17	<0.001
Lactose (%)	4.65	4.38	0.03	<0.001
Lactose yield (g/d)	527.83	299.33	10.22	<0.001

[1]Incomplete milking was obtained by stopping the milking at a time when approximately 30% of milk remained in the half gland. Data are presented as LSM of the whole treatment period.

ment × time $P = 0.07$; Supplemental Figure S2; https://data.mendeley.com/datasets/txvr8jzy4k/1; Lacasse, 2022). Fat yield ($P < 0.001$), protein yield ($P < 0.001$), and lactose yield ($P < 0.001$) were all lower in the INC quarters (Table 2).

Gene Expression

The expression of several genes related to milk synthesis (*CSN2*, $P = 0.02$; *LALBA*, $P < 0.01$; and *ACACA*, $P = 0.01$) was lower in the INC quarters than in the COMP quarters (Table 3). The expression of the proapoptotic gene *BAX* ($P < 0.01$) was higher in the INC quarters than in the COMP quarters, whereas expression of the antiapoptotic gene *BCL2* was unaffected ($P = 0.27$). Accordingly, the *BAX:BCL2* ratio was greater in the quarters milked incompletely ($P = 0.02$). The expression of the short isoform of *PRLR* gene tended to be lower ($P = 0.08$) in the INC quarters. The expression of *STAT5A* and *STAT5B* tended to be lower ($P = 0.08$ and $P = 0.06$, respectively) in the INC quarters than in the COMP quarters. The expression of *SOCS3* was unaffected by the treatments ($P = 0.17$).

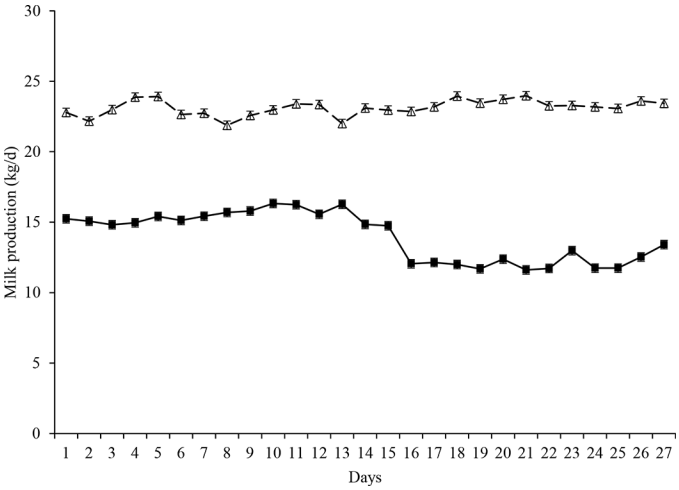

Figure 1. Milk production of half glands milked completely (Δ, n = 8) or incompletely (■, n = 8). Incomplete milking was obtained by stopping the milking at a time when approximately 30% of milk remained in the half gland. This time was re-evaluated at the end of the second week. Data are presented as LSM ± SEM, and milk production during the week before the onset of treatment was used as a covariable.

PRLR and STAT Protein and Activation Levels

In mammary gland biopsy samples, the abundance of both short and long isoforms of the PRLR were unaffected by the treatment; however, the ratio of $PRLR_{short}$ and $PRLR_{long}$ was lower ($P = 0.05$) in the quarters milked incompletely (Table 4). The amount of phosphorylated (activated) and nonphosphorylated forms of STAT3 and STAT5 were similar in both halves of the gland ($P > 0.1$, Table 5).

Table 3. Milk fat-extracted mRNA levels from half glands of cows milked completely (n = 8) or incompletely (n = 8) for 4 wk[1]

Gene[2]	Complete milking	Incomplete milking	SEM	*P*-value
CSN2	3.10	2.03	0.31	0.02
LALBA	3.27	1.90	0.29	<0.01
ACACA	3.11	2.01	0.29	0.01
BAX	0.66	0.90	0.05	<0.01
BCL2	0.77	0.90	0.08	0.27
BAX:BCL2	0.87	1.02	0.04	0.02
$PRLR_{long}$	2.91	2.29	0.32	0.18
$PRLR_{short}$	3.27	2.13	0.44	0.08
SOCS3	0.69	0.32	0.18	0.17
STAT5A	2.00	1.47	0.21	0.08
STAT5B	2.03	1.41	0.22	0.06

[1]Incomplete milking was obtained by stopping the milking at a time when approximately 30% of milk remained in the half gland; data are presented as LSM of the ratio of each gene relative to the geometric mean of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and ribosomal protein S24 (*RPS24*).

[2]*CSN2* = β-casein; *LALBA* = α-lactalbumin; *ACACA* = acetyl-coenzyme A (CoA) carboxylase α; *BAX* = BCL2-associated X protein; *BCL2* = B-cell CLL/lymphoma 2; *BAX:BCL2* = ratio of *BAX* to *BCL2*; $PRLR_{long}$ = prolactin receptor, long isoform; $PRLR_{short}$ = prolactin receptor, short isoform; *SOCS3* = suppressors of cytokine signaling 3; *STAT5A* = signal transducer and activator of transcription 5A; *STAT5B* = signal transducer and activator of transcription 5B.

Table 4. Prolactin receptor (PRLR) short and long isoform levels in mammary gland biopsies collected from rear quarters milked completely (n = 8) or incompletely (n = 8) at the end of the experimental period (wk 4)[1]

Variable[2]	Complete milking	Incomplete milking	SEM	*P*-value
$PRLR_{long}$[3]	6.72	6.79	0.03	0.19
$PRLR_{short}$	7.17	7.14	0.04	0.68
$PRLR_{short}$:$PRLR_{long}$	3.24	2.81	0.13	0.05

[1]Incomplete milking was obtained by stopping the milking at a time when approximately 30% of milk remained in the half gland; data are presented as LSM.

[2]$PRLR_{long}$ = prolactin receptor, long isoform; $PRLR_{short}$ = prolactin receptor, short isoform.

[3]Relative PRLR protein level (log_{10}, arbitrary units).

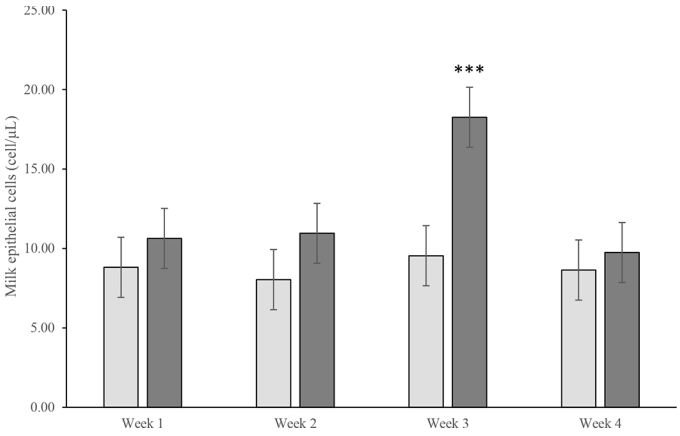

Figure 2. Epithelial cell concentration in the milk of half glands milked completely (light gray, n = 8) or incompletely (dark gray, n = 8). Incomplete milking was obtained by stopping the milking at a time when approximately 30% of milk remained in the half gland. This time was re-evaluated at the end of the second week. ***$P \leq 0.001$. Data are presented as LSM ± SEM.

Mammary Epithelial Cells in Milk and Mammary Gland Functional Capacity

The concentration of mammary epithelial cells in the milk was greater ($P = 0.04$) in the INC half gland than in the COMP half gland. However, we also observed a trend for an interaction time × treatment ($P = 0.06$) with an increase of epithelial cells in wk 3 (Figure 2). The total number of epithelial cells exfoliated in milk was not affected by treatments ($P = 0.34$; data not shown).

The mammary gland functional gland capacity was evaluated before and after the end of the treatment period (2 wk after, in wk 6). At the start of the experiment, we observed no difference in functional capacity ($P = 0.37$) between the half glands, averaging 16.2 ± 0.40 kg and 17.24 ± 0.40 kg for the COMP and INC half glands, respectively. At the end of the experiment, functional capacity was lower ($P = 0.01$) in the INC half glands, averaging 15.22 ± 0.40 kg and 12.61 ± 0 0.40 kg in the COMP and INC half glands, respectively as shown in Figure 3.

DISCUSSION

In this experiment, mid-lactation dairy cows were incompletely milked according to a half-udder design, a powerful tool for exploring the autocrine-paracrine modulation of lactation. Here, our objective was to further analyze the effect of local factors, such as incomplete milking on mammary epithelial cell activity, number, and responsiveness to blood PRL.

During the treatment period, milk production of the INC half glands declined, whereas that of the COMP half glands was maintained. This was also observed in other incomplete milking experiments involving a half-udder design. For example, Penry et al. (2017) and Kuehnl et al. (2019) both reported an overall decrease of 25% and 27%, respectively, in milk production rate when the quarters were incompletely milked. Similarly, differential milking in a half gland model of milking once daily (1×) versus thrice daily (3×) also induces a decline of milk production in half glands milked 1× only (Bernier-Dodier et al., 2010; Boutinaud et al., 2013; Toledo et al., 2020). Nevertheless, although a reduction of milking frequency induces a gradual decline in milk production, the mammary gland adapts quickly to incomplete milking, with milk production reaching a new plateau within a few days. So, as expected, incomplete milking had a negative effect on milk yield; however, more frequent adjustments would have been required to maintain a constant decline in milk production.

The effect of incomplete milking on milk production may be due to a reduction in the number of secretory cells or to a reduction in secretory cell activity. We observed a reduced expression of genes related to milk synthesis such as *CSN2*, *LALBA* and *ACACA*, which encode β-CN, α-LA, and acetyl-CoA carboxylase, respectively. This suggests a decrease in epithelial cell activity. This modulation by milking frequency (as illustrated by changes in the expression of genes involved in milk synthesis) was also present in other studies, including some that used a differential milking design (Bernier-Dodier et al., 2010; Boutinaud et al., 2019; Toledo et al., 2020). These results indicate that a reduction of secretory activity is involved in the reduction of milk yield by incomplete milking.

As stated previously, milk production is dependent on the activity of the cells as well as the number of secretory cells in the gland. Mammary gland functional capacity, an indirect measurement of gland size (Davis et al., 1998), was decreased in INC quarters, suggesting a

Table 5. Signal transducer and activator of transcription (STAT)3 and STAT5 levels in mammary gland biopsies collected from rear quarters milked completely (n = 8) or incompletely (n = 8) at the end of the experimental period (wk 4)[1]

Variable	Complete milking	Incomplete milking	SEM	*P*-value
Phosphorylated STAT3	6.17	6.10	0.39	0.91
Total STAT3	15.42	14.42	2.79	0.81
STAT3 activation[2] (%)	41.98	47.73	4.53	0.40
Phosphorylated STAT5	6.66	6.08	0.76	0.60
Total STAT5	30.40	31.86	3.18	0.75
STAT5 activation[3] (%)	21.98	20.27	2.23	0.61

[1]Incomplete milking was obtained by stopping the milking at a time when approximately 30% of milk remained in the half gland; data are presented as LSM and as $\log_{10}$ (arbitrary units).

[2]Calculated as (phosphorylated STAT3/total STAT3) × 100.

[3]Calculated as (phosphorylated STAT5/total STAT5) × 100.

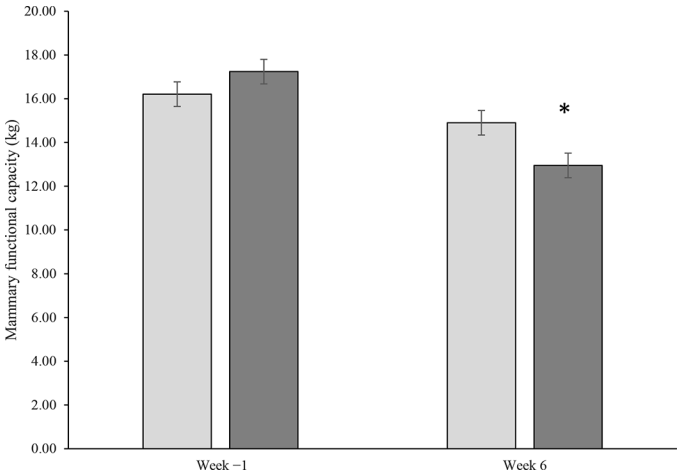

Figure 3. Mammary gland functional capacity of half glands milked completely (light gray, n = 8) or incompletely (dark gray, n = 8). Incomplete milking was obtained by stopping the milking at a time when approximately 30% of milk remained in the half gland. This time was re-evaluated at the end of the second week. *$P \leq 0.05$. Data presented as LSM ± SEM.

loss of mammary tissue in those quarters. Measurement of functional udder capacity can be erroneous if the measurement is not made when the gland is completely full. In our case we made the measurement after restoration of secretion rate 12 d after the end of treatments. At that time the udders were dripping milk, indicative of a full gland at the time of measurement. By 12 d, the secretion rate of active cells in the udder would have been restored so the difference in capacity will represent the loss of capacity through loss of secretory cells (alveoli). The total yield of epithelial cells in milk was not affected by incomplete milking, whereas the concentration of epithelial cells in milk was increased; this was offset by the reduction in milk production. However, during involution and throughout the lactation, epithelial cells are shed from the epithelium into the milk, and the concentration of these cells increases as lactation progresses and as milk yields decrease (Boutinaud and Jammes, 2002). This loss of cells in the INC glands was associated with the upregulation of the proapoptotic gene *BAX* and with a *BAX:BCL2* ratio that is greater in the INC half gland than in the COMP half gland, suggesting greater apoptosis in the secretory cells in addition to a loss of activity. Similarly, Toledo et al. (2020) reported that lower milking frequency was associated with losses of mammary functional capacity, a greater *BAX:BCL2* ratio, and reduced milk production, which was still present several weeks after differential milking had stopped. Therefore, incomplete milking for several weeks reduces the number of epithelial cells, most likely by apoptosis, which contributes to the reduction of milk production.

Beyond the fact that incomplete milking affects milk production by reducing mammary cells activity and number, determining the mechanism by which these effects were obtained is also of interest. As indicated earlier, differential milking experiments have shown that PRL binding capacity can be altered such that PRL responsiveness can be modulated differently in each gland (McKinnon et al., 1988; Bernier-Dodier et al., 2010; Toledo et al., 2020). Throughout the whole treatment period in the present study, only a trend for lower expression of the short isoform of the PRL receptor was observed. However, milk production data indicate that the mammary gland adjusts quickly to incomplete milking, plateauing after only a few days. Therefore, results obtained during the plateau period may fail to indicate an effect. Accordingly, when weekly gene expression data were considered, the expression of *STAT5A*, *STAT5B*, and the long and short isoforms of *PRLR* were significantly lower in the samples collected from INC quarters after milking times were adjusted. Although we cannot confirm the hypothesis that modulation of PRL responsiveness is involved in the effect of incomplete milking, we cannot reject it either, because the experimental design was not optimal.

Tight junctions between mammary epithelial cells are important in maintaining secretory cell activity and viability (Stelwagen and Singh, 2014). In the present study, we observed a decrease in the lactose percentage of milk from the INC half gland throughout the entire experimental period. This result is consistent with those obtained previously by other studies in which lactose percentage was also reduced by incomplete milking (Wheelock et al., 1965; Penry et al., 2017; Kuehnl et al., 2019). A decrease in milk lactose is commonly observed in situations where there is a loss of tight junction integrity, such as milk stasis (Stelwagen et al., 1994), mastitis (Bouchard et al., 1999), or once-a-day

milking (Bernier-Dodier et al., 2010; Alex et al., 2015), which suggests that incomplete milking decreases the integrity of tight junctions. In goats, intramammary infusions of a calcium chelator (EGTA) induced a disruption in the mammary epithelium tight junctions, a reduction of milk lactose concentration, and a reduction of milk secretion (Stelwagen et al., 1995; Ben Chedly et al., 2010). Ben Chedly et al. (2010) also reported an increase of apoptosis in mammary glands infused with EGTA. Therefore, it is possible that the effect of incomplete milking on the mammary gland is due in part to a disruption of the tight junctions.

In conclusion, the decrease in milk yield induced by incomplete milking is rapid and may be associated with a decrease in mammary epithelial cells activity as well as with a decrease in the number of secretory epithelial cells, resulting from the induction of apoptosis. Although the underlying mechanism remains to be determined, it is likely that an increase in the permeability of the tight junctions in the mammary epithelium is involved. Additional evidence is needed to determine whether the mammary gland responsiveness to the PRL galactopoietic signal is modulated by incomplete milking.

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