Effects of intramammary infusions of casein hydrolysate, ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid, and lactose at drying-off on mammary gland involution

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ABSTRACT

The transition from the lactation to the dry period in dairy cows is a period of high risk for acquiring new intramammary infections. This risk is reduced when involution of mammary glands is completed. Consequently, strategies that accelerate the involution process after drying-off could reduce the incidence of mastitis. The objective of this study was to assess the effect of 3 different treatments on mammary gland involution. Each quarter of 8 Holstein cows in late lactation was randomly assigned at drying-off to an intramammary infusion of casein hydrolysate (CNH; 70 mg), ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA; 5.7 g), lactose (5.1 g), or saline 0.9% (control) solutions. Milk samples were collected on the last 2 d before and 1, 3, 5, 7, 10, and 14 d after the last milking for determining concentrations of mammary gland involution markers. Lactoferrin, somatic cell counts (SCC), BSA, and Na^+ concentrations, as well as matrix metalloproteinase-2 and matrix metalloproteinase-9 activities gradually increased in mammary secretions during the first 2 wk following the last milking, whereas milk citrate and K^+ concentrations decreased. As involution advanced, the $\text{Na}^+:\text{K}^+$ ratio increased, whereas the citrate:lactoferrin ratio decreased. Compared with mammary secretions from control quarters, mammary secretions of quarters infused with CNH had higher SCC on d 1, 3, 5, and 7, and greater BSA concentrations on d 1, 3, and 5. Similarly, the CNH treatment induced a faster increase in lactoferrin concentrations, which were greater than in milk from control quarters on d 3, 5, and 7 after drying-off. Milk citrate concentrations were unaffected by CNH but the citrate:lactoferrin ratio was lower in CNH-treated quarters on d 3 and 5 than in control quarters. Moreover, CNH treatment hastened the increase in Na^+ concentration and in the $\text{Na}^+:\text{K}^+$

ratio on d 1. Infusion of CNH also led to an increase in proteolytic activities, with greater matrix metalloproteinase 9 activities on d 1 and 3. The EGTA infusion increased SCC above that of control quarters on d 1 and 3 but it had no effect on the other parameters. Lactose infusion had no effect on any of the involution markers. In this study, intramammary infusions of CNH were the most efficient treatment to accelerate mammary gland involution, suggesting a potential role of CNH as a local milk secretion inhibitor during milk stasis.

Key words: dairy cow, mammary gland involution, casein hydrolysate

INTRODUCTION

The transition from a lactating to a nonlactating state represents a challenge for modern dairy cows. Although milk is not removed anymore, the mammary gland continues to synthesize milk for a few days, leading to an engorgement of the gland and to milk leakage, facilitating the entry of microorganisms into the udder through the teat canal. Accordingly, the risk of new IMI in dairy cows is enhanced during this period, especially for high-yielding cows (Rajala-Schultz et al., 2005). It has been estimated that 16.7% of quarters that were bacteriologically negative before cessation of milking become infected during the dry period, regardless of antibiotic treatments (Dingwell et al., 2002).

Mammary gland involution is a remodeling process that starts as soon as 2 d after cessation of milking in dairy cows (Holst et al., 1987) and through which the gland returns to a nonlactating state. During this process, among other physiological events, there is a reduction in the synthesis and secretion of milk components, recruitment of immune cells, and antibacterial proteins such as immunoglobulins and lactoferrin (Fleet and Peaker, 1978; Sordillo et al., 1987; Monks et al., 2002), as well as an increase in enzyme activities, such as those of plasmin, plasminogen activator (PA), and matrix metalloproteinases (MMP; Talhouk et al., 1992; Politis, 1996). Another characteristic of

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mammary gland involution is the increase in the permeability of the tight junctions that link the mammary epithelial cells together (Nguyen and Neville, 1998). Tight junctions are located at apical sides of epithelial cells and make the mammary epithelium impermeable to paracellular transport between interstitial fluid and milk. Tight junctions are strongly closed during lactation and become permeable during involution, thereby causing a change in mammary gland fluid composition (Nguyen and Neville, 1998), which can be used to measure the involution rate (Shamay et al., 2003).

When involution is advanced, the mammary gland is more resistant to new IMI (Oliver and Smith, 1982). Nonnecke and Smith (1984) have reported that inhibition of *Escherichia coli* growth by whey increased significantly during the dry period and the inhibition was maximal in wheys collected on d 15 of the dry period. In addition, treatments accelerating the rate of involution, such as intramammary infusions of colchicine, endotoxin, or a combination of both, reduced the rate of new IMI during the first week of involution (Oliver and Smith, 1982). Consequently, it would be interesting to find certain strategies which could hasten the mammary gland involution process.

Intramammary infusions of casein hydrolysates (CNH) in dairy goats and dairy cows decrease milk synthesis (Silanikove et al., 2000; Shamay et al., 2002) and increase tight junction permeability (Shamay et al., 2002, 2003). In goats, intramammary infusion of the calcium chelator ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) also affected the integrity of tight junctions between mammary epithelial cells and milk secretion (Stelwagen et al., 1995; Ben Chedly et al., 2010). In Madin-Darby canine kidney (MDCK) cells, addition of EGTA to the culture milieu led to a decrease in transepithelial electrical resistance and disrupted the continuity of the tight junction network (Rothen-Rutishauser et al., 2002). In vivo, infusions of EGTA into goat mammary glands caused a decrease in milk potassium concentration, an increase in milk sodium concentration, and an increase in blood lactose concentration, as well as a decrease in milk secretion (Stelwagen et al., 1995). Further, Ben Chedly et al. (2010) have reported that intramammary infusions of lactose led to a disruption of the mammary epithelium. Therefore, the objective of this study was to assess the effect of intramammary infusions of CNH, EGTA, and lactose on mammary gland involution in dairy cows. To measure the extent of involution, parameters that are known to be affected during involution were measured, such as SCC; lactoferrin, BSA, citrate, Na^+ , and K^+ concentrations; and MMP activities.

MATERIALS AND METHODS

Animals and Experimental Design

The experiment was conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 1993). Eight first- to fourth-parity Holstein dairy cows in late lactation (373 ± 25 DIM at drying-off), housed in the Howard Webster Center of McGill University (Sainte-Anne-de-Bellevue, QC, Canada), were used. The animals were housed in a tie-stall barn and were fed a late-lactating diet before drying-off and hay after drying-off. One week before drying-off, SCC was measured and bacterial cultures were performed for each quarter. The cows whose quarters were not infected by bacteria and contained less than 200 somatic cells/ μL were selected. Cows were producing 21.2 ± 2.3 kg of milk at drying-off.

After the last milking (d -1), the 4 quarters of each cow were randomly assigned to 1 of the following intramammary infusions: a 50-mL solution of 0.9% saline as a control, a 50-mL solution of distilled water containing 5.7 g of EGTA (pH = 8), a 50-mL solution of distilled water containing 5.1 g of lactose, or a 50-mL solution of saline 0.9% containing 70 mg of CNH (Volcani Center Institute of Animal Science, Bet Dagan, Israel). Casein hydrolysates were prepared as described by Shamay et al. (2003). All solutions were prepared under sterile conditions and teats were scrubbed with gauzes soaked in 70% ethanol before infusion through the teat canal using a thin, rounded plastic needle.

Two days before cessation of milking (d -2 and -1 relative to drying-off), 50 mL of milk was manually collected from each quarter at the a.m. milking. During the dry period, mammary secretions (50 mL) were manually collected on the morning on d 1, 3, 5, 7, 10, and 14 after drying-off. Milk samples were samples collected before drying-off, whereas mammary secretion samples were samples collected from the gland after drying-off. After the last sampling, on d 14, cows were infused with antibiotics (Novodry Plus; Pfizer Canada Inc., Kirkland, QC, Canada) and a teat sealant (Orbeseal; Pfizer Canada Inc.). After milk and mammary secretion collection, samples were mixed by inversion and 2 mL was used to measure SCC. The remaining fluid was skimmed by centrifugation ($1,900 \times g$ for 15 min at 4°C) and stored at -20°C until analyses of lactoferrin, citrate, BSA, Na^+ , and K^+ concentrations, as well as determination of gelatinase activities.

SCC in Milk and Mammary Secretions

Somatic cell count was analyzed with an automatic cell counter (DeLaval International AB, Tumba, Swe-

den). Mammary secretion samples were diluted with commercial skimmed microfiltered milk until SCC reached a value between 100 and 200 cells/ μ L.

Lactoferrin and Citrate Concentrations in Milk and Mammary Secretions

The concentration of lactoferrin in skim milk and mammary secretions was measured by ELISA using a commercial bovine lactoferrin ELISA quantitation set (Bethyl Laboratories Inc., Montgomery, TX) according to the manufacturer's instructions. Milk and mammary secretion samples were diluted to obtain a concentration of lactoferrin between 31.25 and 500 ng/mL. The absorbance for each sample was measured at 450 nm using a SpectraMax 250 microplate reader (Molecular Devices LLC, Sunnyvale, CA). The intra- and interassay coefficients of variation were 3.0 and 3.4%, respectively.

The concentration of citrate in skim milk and mammary secretions was determined by an enzymatic assay using a commercial citrate assay kit (Megazyme International Ireland, Bray, Ireland). Before analysis, the skimmed samples were deproteinized by adding an equal volume of cold 1 M perchloric acid. After centrifugation ($1,500 \times g$ for 10 min at 4°C), they were neutralized with 1 M KOH. The citrate concentration was then measured according to the manufacturer's recommendations. The intra- and interassay coefficients of variation were 1.6 and 3.2%, respectively.

The molar citrate:lactoferrin ratio was calculated by dividing the molar concentration of citrate [citrate concentration/citrate molecular weight (192.1 g/mol)] with the molar concentration of lactoferrin [lactoferrin concentration/lactoferrin molecular weight (78,056 g/mol)].

BSA Concentration in Milk and Mammary Secretions

The concentration of BSA in milk and mammary secretions was analyzed by a colorimetric assay as previously described by Bouchard et al. (1999), with some modifications. Briefly, 200 μ L of skim milk or mammary secretion was mixed with 450 μ L of distilled water and 450 μ L of a solution containing 1 volume of 1.2 mM of bromocresol green dissolved in 5 mM NaOH, 3 volumes of 0.2 M succinic acid (pH 4.0), and 0.8% Brij 35 detergent. After mixing by inversion and centrifugation at $1,900 \times g$ for 10 min at room temperature, 150 μ L of the supernatant was added to a 96-well microplate and the optical density was read at 640 nm using a SpectraMax 250 microplate reader.

Na⁺ and K⁺ Concentrations in Milk and Mammary Secretions

The concentrations of Na⁺ and K⁺ in skim milk and mammary secretions were analyzed by flame atomic

emission spectrometry. Commercial Na⁺ and K⁺ reference standard solutions (Fisher Scientific, Ottawa, ON, Canada) were used to establish standard curves, ranging from 0.2 to 1 mg/mL for Na⁺ and from 0.5 to 2 mg/mL for K⁺. Samples were diluted in a solution containing 0.02 N HCl and 0.5 g/L CsCl and concentrations of Na⁺ and K⁺ were measured by an atomic absorption spectrophotometer (Analyst 300 Spectrometer; PerkinElmer Instruments, Woodbridge, ON, Canada). The intra- and interassay coefficients of variation were 0.35 and 0.44%, respectively, for Na⁺ and 0.19 and 0.85%, respectively, for K⁺. The molar Na⁺:K⁺ ratio was calculated by dividing the molar concentration of Na⁺ [Na⁺ concentration/Na⁺ molecular weight (23.0 g/mol)] with the molar concentration of K⁺ [K⁺ concentration/K⁺ molecular weight (39.1 g/mol)].

Gelatinase Activities of Milk and Mammary Secretion

Gelatinase activity in milk and mammary secretions was analyzed by performing gelatin zymography. Briefly, gelatinases were separated on a 10% polyacrylamide gel containing 2 mg/mL of gelatin. Two microliters of skim milk from d -2 and -1, and 1 μ L of skimmed mammary secretions from d 1, 3, 5, and 7 or 0.5 μ L of skimmed mammary secretions from d 10 and 14 were used for migration. Migration was performed at 4°C for 20 min at 90 V and for 150 min at 150 V. The gels were then washed with gentle shaking for 30 min in 100 mL of 2.5% Triton X-100 and washed for 30 min in 50 mL of developing buffer (50 mM Tris base, 200 mM NaCl, 5 mM CaCl₂, and 0.02% Brij 35) before incubation for 22 h at 37°C in developing buffer. The gels were stained with a 50-mL solution containing 0.5% Coomassie blue G-250, 40% methanol, and 10% acetic acid, and then washed with gentle shaking 3 times for 30 min with a 50-mL solution containing 50% methanol, 10% acetic acid, and 40% distilled water. Pictures of the gels were taken using a FluorChem SP imaging system (Alpha Innotech Corp., San Leandro, CA) and integrated density of each band (pixel intensity $\times$ area) was analyzed with AlphaEaseFC software (Alpha Innotech). Due to the factor of dilution, integrated density of bands from d 1, 3, 5, and 7, and bands from d 10 and 14 were multiplied by 2 and 4, respectively.

Statistical Analysis

Data were analyzed by ANOVA using PROC MIXED of SAS (SAS Institute Inc., Cary, NC). Time was used as a repeated effect and quarter (treatment) was used as the subject. When variances were not homogeneous, data were log₁₀-transformed before analyses. Differences were considered statistically significant at $P \leq$

Figure 1. Somatic cell count in milk and mammary secretions (cells/ μ L) from quarters of dairy cows ($n = 8$) infused with a casein hydrolysate (CNH), glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA), lactose, or saline (control) at drying-off. The time of the intramammary infusions is represented by the black arrow. Data are presented as least squares means $\pm$ SEM of $\log_{10}$ -transformed values and significant differences relative to the control are indicated as follows: * $P < 0.05$; ** $P < 0.01$.

0.05. Orthogonal contrasts were performed to compare the effect of each treatment to control.

RESULTS

Before drying-off, SCC; lactoferrin, citrate, BSA, Na^+ , and K^+ concentrations; and MMP activities were similar for all treatments. Somatic cell counts increased ($P < 0.001$) after drying-off and an interaction was observed between time and treatments ($P < 0.001$; Figure 1). Intramammary CNH infusion induced a faster increase in SCC, which was higher ($P < 0.05$) than that of control quarters from d 1 to d 7. Infusion of EGTA increased SCC above that of control quarters at d 1 and 3 ($P < 0.05$). Lactose infusion did not affect SCC at any time.

Similar to SCC, concentrations of BSA in mammary secretions increased ($P < 0.001$) after drying-off and an interaction was observed between time and treatments ($P < 0.001$; Figure 2). Intramammary infusions of CNH hastened the increase in BSA concentrations in mammary secretions, which were higher ($P < 0.01$) than those of control quarters from d 1 to 5 and tended to be higher on d 7 ($P = 0.09$). The EGTA and lactose infusions did not affect BSA concentrations.

Lactoferrin concentrations increased ($P < 0.001$) gradually in mammary gland secretions, and a trend existed for an interaction between time and treatments ($P = 0.08$; Figure 3). Once again, CNH intramammary infusions led to a faster increase in lactoferrin concentrations in mammary secretions, which were higher than those of control quarters from d 3 to 7 ($P < 0.05$). In contrast, concentrations of citrate in mammary secretions varied over time but were not affected by any of the treatments (data not shown). The citrate:lactoferrin ratio decreased ($P < 0.001$) during involution, and a trend was observed for an interaction between time and treatments ($P = 0.08$; data not shown). This ratio was lower in CNH-treated quarters on d 3 and 5 ($P < 0.05$) than in control quarters. The EGTA and lactose intramammary infusions did not affect concentrations of lactoferrin and citrate or the molar citrate:lactoferrin ratio.

Potassium and sodium concentrations in mammary gland fluids exhibited 2 different patterns. After cessation of milking, K^+ concentrations in mammary secretions decreased ($P < 0.001$; data not shown), whereas Na^+ concentration increased ($P < 0.001$; data not shown). Treatments did not affect K^+ concentration, but an interaction existed between time and treatments

Figure 2. Bovine serum albumin concentration in milk and mammary secretions from quarters of dairy cows ($n = 8$) infused with a casein hydrolysate (CNH), glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA), lactose, or saline (control) at drying-off. The time of the intramammary infusions is represented by the black arrow. Data are presented as least squares means $\pm$ SEM and significant differences relative to the control are indicated as follows: ** $P < 0.01$.

($P < 0.001$) for Na^+ concentration. Infusion of CNH hastened the increase in Na^+ concentration, which was higher in CNH-treated quarters than in control quarters on d 1 ($P < 0.05$) and tended to be higher on d 3 ($P = 0.06$). The $\text{Na}^+:\text{K}^+$ ratio increased ($P < 0.001$) during involution and an interaction was observed between time and treatments ($P < 0.05$; data not shown). The ratio was higher in CNH-treated quarters on d 1 ($P < 0.05$) than in control quarters. The Na^+ and K^+ concentrations as well as $\text{Na}^+:\text{K}^+$ ratio were not affected by EGTA or lactose infusions.

Milk and mammary secretion proteolytic activities were assessed by measuring gelatinase activity by zymography. Activities of MMP-2 (Figure 4A) and MMP-9 (Figure 4B) increased ($P < 0.001$) progressively after drying-off. Treatments did not affect MMP-2 activity, but an interaction was observed between time and treatments ($P < 0.001$) for MMP-9 activity. The increase in MMP-9 activity after cessation of milking was hastened by CNH treatment. Indeed, MMP-9 activity was higher ($P < 0.001$) in mammary secretions from CNH-treated quarters compared with those from control quarters on d 1 and 3, and tended to be higher on d 5 ($P = 0.06$). Intramammary infusion of EGTA

increased MMP-9 activity on d 3 ($P < 0.05$). Lactose treatment did not affect MMP-9 activity.

DISCUSSION

The objective of the present study was to test whether intramammary infusions of CNH, EGTA, and lactose, which may increase tight junction permeability, could affect the speed of the mammary gland involution process. Different cellular or molecular markers related to mammary gland involution were measured to assess its rate of occurrence.

One of the major characteristics of mammary gland involution is epithelium tight junction impairment. Tight junctions, which seal mammary epithelial cells at their apical sides, allow the delimitation between 2 different compartments. In the lumen of the alveoli above the apical side of the epithelium, milk is rich in K^+ and milk-specific proteins such as caseins and α -LA, and low in Na^+ and blood components such as BSA (Nguyen and Neville, 1998). Conversely, the interstitial fluid below the basolateral side of the epithelium is rich in Na^+ and BSA, and low in K^+ and milk-specific proteins (Nguyen and Neville, 1998). During an established lactation,

Figure 3. Lactoferrin concentration in milk and mammary secretions (ng/mL) from quarters of dairy cows ($n = 8$) infused with a casein hydrolysate (CNH), glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), lactose, or saline (control) at drying-off. The time of the intramammary infusions is represented by the black arrow. Data are presented as least squares means $\pm$ SEM of $\log_{10}$ -transformed values and significant differences relative to the control are indicated as follows: * $P < 0.05$; ** $P < 0.01$.

mammary tight junctions are closed and prevent paracellular transport between mammary epithelial cells (Fleet and Peaker, 1978). In contrast, tight junctions become leaky during involution, and milk components can pass through the epithelium to the interstitial fluid and vice versa (Fleet and Peaker, 1978). Mammary tight junctions are impaired as soon as 18 and 21 h of milk stasis in cows and goats, respectively (Stelwagen et al., 1994, 1997). This opening explains the increase in milk Na^+ and BSA concentrations and the decrease in milk K^+ concentration. In our study, CNH treatment hastened the increase in BSA concentration until 5 d after cessation of milking. Although CNH infusion did not affect K^+ concentration, this treatment hastened the increase in Na^+ concentration and in $\text{Na}^+:\text{K}^+$ ratio on d 1. Therefore, we suggest that mammary tight junction integrity was compromised more rapidly in the CNH-treated quarters than in the control quarters after drying-off. These results are concordant with previous studies in which CNH intramammary infusions were performed in lactating goats and late-lactation cows. Indeed, repeated doses of CNH infused in lactating goats (4 doses of 300 mg) induced almost a 5-fold increase in milk BSA and Na^+ concentrations and a

rapid decrease in milk K^+ concentration (Shamay et al., 2002). In cows, repeated doses (6 doses of 67.5 mg) of CNH intramammary infusions before drying-off mimicked the involution process by increasing milk BSA and Na^+ concentrations and by decreasing milk K^+ concentration (Shamay et al., 2003). In our study, we demonstrated that a single dose of 70 mg of CNH infused at drying-off was able to increase tight junction permeability in the cow. In contrast, infusions of EGTA and lactose did not affect BSA, Na^+ , and K^+ concentrations in mammary secretions. Therefore, CNH infusion was the most effective treatment to affect the integrity of mammary tight junctions.

Changes in lactoferrin and citrate concentrations represent 2 additional indicators for mammary gland involution. Lactoferrin concentrations naturally increase in mammary secretions during the first week of involution, reach a peak around 14 d, and remain high throughout the dry period (Nonnecke and Smith, 1984; Sordillo et al., 1987). Conversely, citrate concentrations and the citrate:lactoferrin ratio decrease during the first 7 d of involution and remain low during the dry period (Sordillo et al., 1987). The fact that citrate concentrations decrease after cessation of milking is indicative of

Figure 4. Matrix metalloproteinase 2 (MMP-2; A) and matrix metalloproteinase 9 (MMP-9; B) activities in milk and mammary secretions from quarters of dairy cows ($n = 8$) infused with a casein hydrolysate (CNH), glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), lactose, or saline (control) at drying-off. The time of the intramammary infusions is represented by the black arrow. Data are presented as least squares means $\pm$ SEM of integrated density values and significant differences relative to the control are indicated as follows: * $P < 0.05$; ** $P < 0.01$.

a reduction of the secretory activity of alveolar cells. In our study, none of the treatments affected citrate concentrations. This may suggest that mammary epithelial cell secretory activity was not affected by the treatments. However, CNH intramammary infusion hastened the increase in lactoferrin and the decrease in citrate:lactoferrin ratio on d 3, 5, and 7, and on d 3 and 5, respectively. These results suggest that mammary involution was accelerated by CNH.

In all the treated quarters, SCC increased after cessation of milking and reached a plateau around d 7 of the dry period. An increase in total leukocyte concentrations in mammary secretions is characteristic of active involution (Hurley, 1989). Sordillo et al. (1987) reported that SCC naturally increases in mammary secretions from the last milk removal to at least d 7 of involution. The increase in SCC observed during mammary gland involution is mainly due to recruitment of immune cells, particularly PMNL and macrophages (Monks et al., 2002). These immune cells are involved in ingestion and clearance of cellular debris and residual milk components, such as casein micelles and lipid droplets (Tatarczuch et al., 1997; Monks et al., 2002; Atabai et al., 2007). The faster increase in SCC with CNH intramammary infusions provides additional evidence that the involution process was accelerated by this treatment.

Another feature of active involution is the increase in the activity of different proteases involved in the degradation of the extracellular matrix and basement membrane components. Matrix metalloproteinase 2 and MMP-9 are 2 proteases that are able to degrade the basement membrane (Matrisian, 1990). Matrix metalloproteinase 2 is mostly produced by the basal myoepithelial cells and epithelial cells and its activity increases during rodent mammary gland involution (Dickson and Warburton, 1992; Talhouk et al., 1992) and during gradual involution in cows (Miller et al., 2006). Matrix metalloproteinase 9 levels in the mammary gland are low during lactation and increase markedly during involution in the mouse (Lund et al., 2000). In the current study, MMP-2 activity increased during the first week of involution but this increase was not affected by any of the treatments. Matrix metalloproteinase 9 activity also increased as involution advanced and CNH treatment hastened this increase on d 1 and 3. This increase in MMP-9 activity could be related to the faster increase in SCC in CNH-treated quarters. Indeed, it has been shown that PMNL represent the main source of MMP during mastitis (Mehrzhad et al., 2005) and that MMP-9 activity is strongly associated with SCC (Miller et al., 2006). Previous studies have reported that activities of PA and plasmin were increased by CNH intramammary infusions in goats (Shamay et al., 2002) and in cows

(Shamay et al., 2003). Plasmin is a proteolytic enzyme naturally present in milk and is predominantly found in its inactive form, plasminogen (Politis, 1996). The conversion of plasminogen into plasmin is regulated by PA and both plasmin and PA activities are increased during milk stasis (Politis, 1996). Moreover, plasmin may activate different pro-MMP to activate MMP such as MMP-9 (Lijnen et al., 1998). Therefore, the increase in MMP activities in CNH-treated quarters in the current study could be related to an increase in plasmin and PA activities.

Globally, the results from this study indicate that CNH infusion can hasten mammary gland involution, but the mechanism is still elusive. Observations from experiments where differential milking frequency (Sorensen et al., 2001; Bernier-Dodier et al., 2010) or unilateral milk stasis (Tremblay et al., 2009) were applied indicate the presence of a local mechanism of regulation for mammary gland involution. Peaker and Wilde (1996) have postulated the existence in milk of a low-molecular-weight protein with an autocrine inhibitory activity on mammary epithelial cells. The increase in proteolytic activity observed during milk stasis (Talhouk et al., 1992; Politics, 1996) could generate casein breakdown products that are able to trigger local inhibition of milk synthesis. It has been shown that the casein breakdown products released by the hydrolysis of β -CN by plasmin and containing O-phospho-L-serine residues could block the activity of K^+ channels located at the apical side of the mammary epithelial cells (Silanikove et al., 2000). The blockade of K^+ channels by CNH could affect mammary epithelial cell secretory activity. Another possible explanation for CNH-induced impairment of mammary tight junctions could be reduced extracellular calcium levels. It has been shown in dairy goats that intramammary infusions of CNH led to a reduction in milk calcium concentrations (Shamay et al., 2002). In mouse mammary epithelial cells in which extracellular calcium was depleted, junction organization was destabilized and junction continuity was disrupted (Pitelka et al., 1983). Intramammary infusion of EGTA, a chelator of calcium, led to a transient disruption of tight junction integrity in goats (Stelwagen et al., 1995). Therefore, CNH could impair the integrity of the mammary epithelium by acting on both K^+ channels and calcium availability.

Intramammary infusion of EGTA appears to weakly stimulate mammary gland involution. In goats, intramammary infusion of EGTA solution led to a decrease in milk secretion and to an impairment of tight junction integrity (Stelwagen et al., 1995; Ben Chedly et al., 2010). The absence of effect of EGTA in the present study on parameters related to tight junction status, such as Na^+ , K^+ , or BSA concentrations, was probably due to the smaller amount of the substance infused.

Stelwagen et al. (1995) observed that an infusion of EGTA reaching a final concentration of 68 mM when diluted with residual milk was able to decrease milk secretion and to cause tight junction disruption. However, those authors reported little effects at a final EGTA concentration of 16 mM. In the present study, 50 mL of a 300 mM EGTA solution was infused per quarter. Assuming that approximately 12% of milk remains in the gland after milking (approximate volume of 0.36 L per quarter; Knight et al., 1994), a concentration of 42 mM was reached in our experiment. Moreover, as the experiment was carried out in dairy cows, the minimal effective EGTA dose to cause mammary tight junction disruption could have been higher than in goats. However, EGTA intramammary infusions led to a faster increase in SCC on d 1 and 3 compared with the control quarters. Moreover, EGTA infusions increased MMP-9 activity on d 3 compared with the control quarters. These results suggest that the low EGTA dose, even if not high enough to impair tight junction integrity, could have increased the recruitment of somatic cells.

No effect of lactose intramammary infusions was observed in this study. This treatment was tested according to studies in the goat by Ben Chedly et al. (2010), who noticed that 4 intramammary infusions of 32 mL of lactose (300 mM) induced a decrease in milk production and milk lactose, milk fat, and milk casein contents. Moreover, α -LA and κ -CN mRNA were less expressed, suggesting that the activity of mammary epithelial cells was reduced. In addition, mammary tight junction integrity seemed to be impaired, as milk K^+ concentration was decreased and milk BSA concentration tended to increase (Ben Chedly et al., 2010). The possible effects of lactose on tight junction integrity were reinforced by an in vitro experiment in which the transepithelial electrical resistance of cultured mammary epithelial cells was decreased by lactose. (Ben Chedly et al., 2010). In the present study, cows received a single 50-mL infusion of a 300 mM lactose solution, leading to a final concentration in the treated quarter of approximately 42 mM. The absence of effect on the parameters related to tight junction integrity or to mammary gland involution might be due to a lower dose of lactose. In the goat, four 32-mL infusions of a 300 mM lactose solution were used (Ben Chedly et al., 2010). Once again, the minimal effective dose for cows would be higher than for goats. However, it remains unclear how lactose, the main osmotic component in milk, could stimulate mammary gland involution.

CONCLUSIONS

Intramammary infusion of CNH was the most efficient treatment to accelerate mammary gland involu-

tion in our study. This treatment could help the gland to be fully involuted more rapidly and, therefore, to be more resistant to IMI. Further studies should be conducted to evaluate the effects of CNH intramammary infusion on the immune system of the dairy cow and on the healthy status of the mammary gland.

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