Anti-inflammatory effects of intramammary infusions of glycyrrhizin in lactating cows with mastitis caused by coagulase-negative staphylococci

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Objective—To determine the anti-inflammatory effects of glycyrrhizin (GL) in lactating cows with mastitis attributable to naturally occurring infection with coagulase-negative staphylococci (CNS).

Animals—12 lactating Holstein cows with mastitis attributable to infection with CNS and 2 healthy cows without mastitis.

Procedure—Clinical signs, number of bacteria in milk, somatic cell count (SCC) in milk, concentrations of α-lactalbumin and lactoferrin in milk, and concentration of histamine in milk were investigated before and after intramammary infusion of GL (6 cows) or antimicrobials (6 cows). Glands of 2 healthy cows were infused with staphylococcal enterotoxin; milk leukocytes were then harvested and incubated with various doses of GL.

Results—In cows infected with CNS that had a low bacterial concentration in milk, infusion of GL alone resulted in significant improvements in swelling, firmness of glands, and number of clots in milk; and it decreased the SCC, but not significantly. Percentage of neutrophils decreased significantly (to < 30%) by 2 days after infusion. Use of lactoferrin as a marker of inflammation in mammary glands revealed a decrease in concentrations, whereas use of α-lactalbumin as a marker of recovery for mammary glands revealed significant increases in concentrations in the GL-infused group. Accompanying these anti-inflammatory effects, a decrease in the concentration of histamine in milk was observed in the GL-infused group. Glycyrrhizin decreased histamine production by milk leukocytes in a concentration-dependent manner.

Conclusions and Clinical Relevance—Infusion of GL may regulate intramammary inflammation through modulation of inflammatory mediators such as histamine. (Am J Vet Res 2003;64:1213–1220)

After invading a mammary gland, pathogenic bacteria adhere to the mammary tissue, grow, and produce bacterial toxins. Coagulase-negative staphylococci (CNS) are commonly isolated from milk during intramammary infections; these organisms cause the production of abnormal milk and a decrease in milk yield. Coagulase-negative staphylococci are capable of interacting with fibronectin-binding protein on the surface of bacteria in mammary tissue and produce mucoid extracellular materials (ie, slime). Therefore, CNS may be a frequent cause of initial intramammary infections. These organisms, which were once considered to be of low pathogenicity, cause mild inflammatory reactions (eg, an increase in somatic cell count [SCC] and local inflammatory reactions) that are limited to the mammary gland without systemic inflammatory effects. Moreover, these bacteria are capable of producing toxins such as enterotoxins and hemolysins.

The migration of leukocytes is elicited by inflammatory mediators produced by cells in response to bacterial toxins. Leukocyte migration into inflamed tissue is definitely attributable to inflammatory mediators such as interleukin (IL)-8, C5a, and leukotriene B4. Histamine, which is also an inflammatory mediator, has little effect on the induction of leukocyte migration but does promote marked swelling of tissue accompanied with vascular permeability. Histamine is also involved in the initial increase in vascular permeability during the early phase of acute inflammation in cows with mastitis. Also, staphylococcal enterotoxin induces the production of histamine through the activation of histidine decarboxylase in mast-cell-deficient mice. To that end, swelling and accumulation of leukocytes in affected glands are caused by CNS infections.

Glycyrrhizin (GL) is a natural compound obtained from the aqueous extract of the root of Leuminosae such as Glycyrrhiza glabra and G uralensis. Glycyrrhizin is composed of 1 molecule of gly
cyrtrinic acid and 2 molecules of glucuronic acid. Glycyrrhizin is known for its regulatory effects on the production of platelet-activating factor and inducible effects on the production of interferon. Glycyrrhizin also exerts inhibitory effects on the phosphorylation of lipocortin, resulting in inhibition of the production of arachidonic metabolites because of the obstruction of arachidonate cascades. Also, GL inhibits histamine release from mast cells in rats.

In the study reported here, CNS were used because the mild mastitis associated with these organisms allowed us to delay initiation of treatment without threatening the health of the cows. Therefore, this study was designed to reveal anti-inflammatory effects of the intramammary infusion of GL on clinical mastitis in cows infected with CNS.

**Materials and Methods**

**Animals**—Twelve cows with mastitis attributable to staphylococcal infection were used in the study. Each cow had only 1 gland with mastitis. The staphylococci were identified as CNS. In addition, 2 healthy cows were used to determine the effects of GL on histamine production in milk leukocytes.

The study was performed on 3 dairy farms that each maintained 80 to 90 milking Holsteins. The farms were located in Miyagi Prefecture in Japan. Clinical mastitis in lactating cows was determined on the basis of an SCC of >500,000 cells/mL of milk and evidence of infection of the mammary gland, such as clots in milk and swelling and firmness of the affected gland. Forty-two glands of 30 lactating cows on the farms had evidence of clinical mastitis during 180 days of observation. Then, a diagnosis of mastitis attributable to staphylococci was made on the basis of >250 colony forming units (CFUs) of staphylococci/mL of milk and a lack of other pathogenic bacteria. Moreover, mastitis caused by naturally acquired CNS infection produced mild inflammation.

Cows were milked twice each day at approximately 8 AM and 4 PM. Of the 12 cows in the study, 2 were ≤50 days of lactation, 7 were between 51 and 150 days of lactation, and 3 were >151 days of lactation.

**Experimental procedure**—After clinical mastitis was detected, milk samples were collected aseptically from the affected glands prior to the regularly scheduled milking. On the day of detection of clinical mastitis, a portion of each milk sample was submitted for microbial culture to investigate whether bacteria in milk were staphylococci. Two days after the onset of mastitis, it was confirmed that 12 mammary glands were infected with only staphylococci and no other pathogenic bacteria. Thus, clinical mastitis was confirmed in all 12 cows by day 3 after the initial onset of intramammary infection.

Twelve infected mammary glands of 12 cows (1 gland/cow) were used to investigate the anti-inflammatory effects of the infusion of GL (6 glands) or antimicrobial (6 glands). The 12 cows were alternately allocated to GL or antimicrobial treatment at the time of initial onset of mastitis.

**Infusion procedure**—Antimicrobial GL was infused after the morning milking. The GL-infused group of 6 cows was infused only once into the infected mammary gland via the teat canal. Preliminary studies revealed that a dose of 400 mg of GL/gland was needed for effective treatment. Therefore, 400 mg of GL (20 mg/mL) was infused once at (mean ± SEM) 3.7 ± 0.3 days after the onset of clinical mastitis. The antimicrobial-infused group of 6 cows was infused once daily for 3 days. Each intramammary infusion consisted of 130 mg of sodium cephalolin, in accordance with the manufacturer’s instructions. The third antimicrobial infusion was administered 5.0 ± 0.7 days after the onset of mastitis.

**Score of clinical signs**—Clinical signs of mastitis were scored to determine the degree of inflammation. Scores were assigned on the basis of the degree of inflammation, as determined for 3 categories. Scores of clinical signs for each affected mammary gland were assigned for swelling of the mammary gland (0, swelling not detected; 1, swelling in part of the mammary gland; 2, swelling throughout the mammary gland), firmness of the mammary gland (0, firmness not detected; 1, firmness in part of the mammary gland; 2, firmness throughout the mammary gland), and clots in milk (0, clots not detected; 1, < 0.5 clots/mL of milk; 2, 0.5 to 4 clots/mL of milk; and 3, ≥5 clots/mL of milk).

**Collection of milk samples**—Milk samples were collected from affected glands of cows before treatment (day 0) and on days 1, 2, 7, and 14 after infusion of GL or antimicrobial.

During the morning milking, the first 3 streams of milk from an affected gland were discarded. Then, 5 mL of milk was collected into a graduated tube; this sample was used to determine the number of clots in the milk. Subsequently, a second milk sample (approx 5 mL of milk) was aseptically obtained from each gland into sterile tubes. Milk samples were collected before infusion of GL or antimicrobial.

In addition, milk samples were collected from 11 healthy glands of 11 clinically normal cows. These samples were used to determine the mean concentration of histamine in normal milk. These samples were considered normal on the basis that each milliliter of milk had an SCC of <200,000 cells, <20 CFUs of bacteria, and <450 µg of lactoferrin.

**Analysis of milk samples**—The number of clots in 5 mL of milk was determined by use of a stainless-steel mesh with a pore size of 2 mm. Therefore, the minimum size of clots used for counting was 2 mm. Subsequently, the number of clots was calculated for 1 mL of milk.

A portion of each milk sample was diluted 10- or 100-fold with sterile saline (0.9% NaCl) solution, and aliquots (0.05 mL) of the undiluted and diluted milk samples were used for microbial culture (in triplicate) on staphylococcus agar No. 110 and 5% (vol:vol) sheep blood containing trypticase soy agar. Plates were incubated at 37°C for 24 hours. After incubation, colonies were placed on slides, and a Gram stain was applied. Staphylococcal colonies were identified on the basis of colony morphology and microscopic evaluation. The number of colonies was enumerated; detection limit for the assay system was 7 CFUs/mL.

To perform the catalase test, staphylococci were cultured on sheep blood containing trypticase soy agar. After completion of the catalase test, staphylococci were identified by use of a staphylococci identification kit.

A portion of each milk sample was used to determine the SCC. The SCC of milk samples was measured by use of fluorescent-based flow cytometry, which was based on the SCC attributable to the specific binding of propidium iodide to DNA.

Determination of the proportion of neutrophils was modified slightly from the method reported by Jensen and Eberhart. Briefly, a 50-mL aliquot of each milk sample was centrifuged at 800 X g for 30 minutes at 4°C.
Supernatant was removed, and pelleted cells were washed twice in 30 mL of PBS solution (pH 7.2). Washed cells were made to adhere to slides by use of microcentrifugation; then the slides were stained with May-Grünwald-Giemsa reagents. After drying, a minimum of 300 cells was identified, and the proportions of various cell types were determined. Cells were classified as neutrophils or others. Proportions of neutrophils were calculated and reported as percentages.

To determine concentrations of lactoferrin and α-lactalbumin in milk samples, a single radial-immunodiffusion (SRID) technique was used. Concentration of lactoferrin was measured by use of a bovine lactoferrin SRID kit. To measure α-lactalbumin, the SRID technique was slightly modified from the method described in another report. Briefly, bovine α-lactalbumin SRID plates were prepared by use of antisemur against bovine α-lactalbumin generated in Japanese white rabbits. The SRID plates consisted of 12% anti-α-lactalbumin serum, 1.8% agarose, and 0.02M Tris-HCl. Aliquots (5 µL) of milk samples or PBS solution (pH, 7.2) containing various concentrations of lactoferrin or α-lactalbumin were incubated on the appropriate SRID plates at 37°C. Concentrations of lactoferrin or α-lactalbumin in the milk samples and PBS solutions (ie, standards) were determined after incubation for 48 and 24 hours, respectively. Standard curves were created for lactoferrin and α-lactalbumin, and concentrations of lactoferrin and α-lactalbumin in milk samples were calculated. Detection limits for lactoferrin and α-lactalbumin in milk samples were 40 and 60 µg/mL, respectively.

Quantification of histamine in milk samples—Preparation of milk samples for the quantification of histamine concentrations was modified slightly from the method reported by Zia et al. Briefly, triplicate aliquots (250 µL/aliquot) of each milk sample were boiled at 100°C for 10 minutes; then, 6.0 mL of 0.3M perchloric acid was added. Samples were centrifuged at 1,750 g for 5 minutes to remove protein and fat, and supernatant was recovered. Supernatant (3 mL) was used to measure the concentration of histamine by use of a fluorometric method established by Endo.

Inhibition of histamine production in milk attributable to intramammary infusion of GL or antimicrobial was determined. Inhibition for each sample was calculated by use of the following equation: percentage inhibition = ([1 – value after infusion]/value before infusion) × 100.

Measurement of histamine production by milk leukocytes—To investigate whether GL regulates histamine production of leukocytes, each of the 4 mammary glands of 2 healthy cows was infused with 20 µg of staphylococcal enterotoxin C (20 µg). Milk samples were obtained 16 hours after intramammary infusion of enterotoxin; the samples were centrifuged, and milk leukocytes were collected. Five hundred microliters of cell suspension (1 × 10⁶ cells/mL) and 500 µL of serum-free medium containing 1% human serum albumin and various concentrations of GL were added to each well of 24-well, plastic tissue culture plates; plates were incubated for 24 hours at 37°C in an atmosphere of 5% CO₂ and 95% air. After incubation, the conditioned medium was collected by centrifugation. Mean ± SEM concentration of histamine for the standard solution was 2.0 ± 0.1 ng/mL. Histamine content of the conditioned medium was measured as described previously.

Statistical analysis—Data were analyzed by use of a 2-tailed t test, except for scores of clinical signs. Scores of clinical signs were analyzed by use of rridit analysis. Data were expressed as mean ± SEM. Values of P < 0.05 were considered significant.

Results Isolation of CNS—Various staphylococcal organisms were isolated during microbial culture. For cows in the GL-infused group, CNS isolated included Staphylococcus xylosus (n = 2), S epidermidis (1), S hyicus (1), S caprae (1), and S hominis (1). For cows in the antimicrobial-infused group, CNS isolated included S xylosus (n = 4) and S sciuri (2).

Changes in clinical score after intramammary infusion of GL or antimicrobial—Although the cows administered GL and antimicrobial infusions were in similar infectious states with regard to the degree of firmness and number of clots prior to infusion, the degree of swelling was significantly more severe in the glands of the GL-infused group, compared with values for the antimicrobial-infused group (Table 1). The GL-infused group had a significant (P < 0.01) improvement for swelling and firmness of glands and the number of clots in milk 2 and 7 days after infusion (Table 2). The antimicrobial-infused group had a transient but significant improvement for swelling of affected glands 2 days after infusion (P < 0.01) and the number of clots in milk 2 and 7 days after infusion (P < 0.01); however, there was not a significant improvement for firmness of glands.

Table 1—Bacterial concentrations in milk in clinical score for cows with mastitis (n = 6 cows/group) prior to treatment by intramammary infusion of a single dose of glycyrrhizin (GL) or 3 doses of an antimicrobial

<table>
<thead>
<tr>
<th>Treatment of gland</th>
<th>Variables for milk samples</th>
<th>Clinical score</th>
<th>Days after onset of</th>
<th>Days after</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treatment of gland</td>
<td>No. of CNS Clot score</td>
<td>SCC (X 10⁶ cells/mL)</td>
<td>Swelling</td>
</tr>
<tr>
<td>GL</td>
<td>Mean ± SEM</td>
<td>2.8 ± 0.2</td>
<td>2.5 ± 0.3</td>
<td>243.8 ± 95.1</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td>2.4–3.7</td>
<td>1–3</td>
<td>56–705</td>
</tr>
<tr>
<td>Antimicrobial</td>
<td>Mean ± SEM</td>
<td>2.8 ± 0.1</td>
<td>2.2 ± 0.4</td>
<td>194.5 ± 55.0</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td>2.4–3.5</td>
<td>1–3</td>
<td>83–447</td>
</tr>
<tr>
<td></td>
<td>*Clot score was determined as follows: 0, clots not detected; 1, &lt; 0.5 clots/mL of milk; 2, 0.5 to 4 clots/mL of milk; and 3, ≥ 5 clots/mL of milk.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n = 6 cows/group prior to treatment by intramammary infusion of a single dose of glycyrrhizin (GL) or 3 doses of an antimicrobial.

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ness alone. We did not detect any adverse reactions after infusion of GL.

Changes in bacterial concentration in milk after intramammary infusion of GL or antimicrobial—Mean concentration of staphylococci in GL-infused glands was not significantly reduced after infusion (Table 3). However, mean concentration of staphylococci in antimicrobial-infused glands was significantly reduced 1, 2, and 7 days after infusion.

Changes in SCC after intramammary infusion of GL or antimicrobial—Mean ± SEM SCC in the GL-infused group was 243.8 ± 95.1 × 10⁴ and 63.0 ± 23.1 × 10⁴ cells/mL on days 0 and 7, respectively, compared with 194.5 ± 55.0 × 10⁴ and 195.0 ± 127.7 × 10⁴ cells/mL in the antimicrobial-infused group (Fig 1). Although SCC in the GL-infused group was not significantly reduced after infusion, mean SCC in this group did decrease slightly 2 days after infusion. Furthermore, the proportion of neutrophils among total somatic cells in milk in the GL-infused group was 65.7 ± 8.2% on day 0. Mean percentage of neutrophils decreased significantly 2 and 7 days after infusion (28.3 ± 10.3 and 23.2 ± 5.9%, respectively), compared with the value for day 0. Similar changes were not detected for the antimicrobial-infused group.

Changes in concentrations of lactoferrin and α-lactalbumin after intramammary infusion of GL or antimicrobial—Mean concentration of lactoferrin in the GL-infused group was 1,173 ± 310 µg/mL on day 0, and it decreased slightly on days 2 and 7 after infusion (888 ± 218 and 771 ± 239 µg/mL, respectively; Fig 2). In the antimicrobial-infused group, mean concentration of lactoferrin was 1,108 ± 351 µg/mL on day 0, and it increased slightly on days 2 and 7 after infusion (1,953 ± 788 and 1,882 ± 705 µg/mL, respectively).

In the GL-infused group, mean concentration of α-lactalbumin was 1,063 ± 108 µg/mL on day 0, but it increased significantly by day 7 (1,373 ± 169 µg/mL; Fig 2). Mean concentration of α-lactalbumin increased slightly, but not significantly, in the antimicrobial-infused group (1,026 ± 256 and 1,141 ± 234 µg/mL on days 0 and 7, respectively).

Table 2—Changes in clinical score in cows with mastitis attributable to CNS (n = 6 cows/group) that were treated by intramammary infusion of a single dose of GL or 3 doses of an antimicrobial

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days after infusion</th>
<th>Clot score*</th>
<th>Swelling of gland†</th>
<th>Firmness of gland‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL</td>
<td>0</td>
<td>2.5 ± 0.3</td>
<td>1.8 ± 0.2</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2.0 ± 0.6</td>
<td>1.3 ± 0.4</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.3 ± 0.3†</td>
<td>0.0 ± 0.0§</td>
<td>0.0 ± 0.0§</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.3 ± 0.3†</td>
<td>0.0 ± 0.0§</td>
<td>0.0 ± 0.0§</td>
</tr>
<tr>
<td>Antimicrobial</td>
<td>0</td>
<td>2.2 ± 0.4</td>
<td>1.2 ± 0.2</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.5 ± 0.6</td>
<td>0.7 ± 0.2</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.5 ± 0.3†</td>
<td>0.5 ± 0.2†</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.8 ± 0.4</td>
<td>0.7 ± 0.3</td>
<td>0.5 ± 0.3</td>
</tr>
</tbody>
</table>

Values reported are mean ± SEM.

Day 0 = Day of GL infusion or first day of infusion of antimicrobial.

†Within each day, values differ significantly (P < 0.05) between groups.

‡Within a treatment, value for that day differs significantly (P < 0.05) from the value for day 0.

*See Table 1 for remainder of key.

Table 3—Changes in bacterial concentration in milk samples obtained from cows with mastitis attributable to CNS (6 cows/group) that were treated by intramammary infusion of a single dose of GL or 3 doses of an antimicrobial

<table>
<thead>
<tr>
<th>Days after infusion</th>
<th>GL (Log10 CFU/mL)</th>
<th>Antimicrobial (Log10 CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.8 ± 0.2</td>
<td>2.8 ± 0.1</td>
</tr>
<tr>
<td>1</td>
<td>2.6 ± 0.2</td>
<td>1.6 ± 0.3†</td>
</tr>
<tr>
<td>2</td>
<td>2.4 ± 0.4</td>
<td>1.7 ± 0.3†</td>
</tr>
<tr>
<td>7</td>
<td>2.6 ± 0.4</td>
<td>1.7 ± 0.3†</td>
</tr>
</tbody>
</table>

Values reported are mean ± SEM.

Day 0 = Day of GL infusion or first day of infusion of antimicrobial.

*Within a treatment, value for that day differs significantly (P < 0.05) from the value for day 0.

†Within each day, values differ significantly (P < 0.05) between groups.

Figure 1—Somatic cell count (SCC; A) and proportion of neutrophils (B) in milk samples obtained from cows with mastitis attributable to infection with coagulase-negative staphylococci (CNS) that were treated by intramammary infusion of a single dose of glycyrrhizin (GL; solid circles) or 3 doses of an antimicrobial (open circles). *Value differs significantly (P < 0.05) from the value for day 0 in the GL-infused group. Day 0 = Day of infusion of GL or first day of infusion of the antimicrobial.
Changes in concentration of histamine after intramammary infusion of GL or antimicrobial—

Mean concentration of histamine in normal milk from 11 glands of 11 healthy cows was 85 ± 9 ng/mL. Mean concentration of histamine 0, 2, and 7 days after infusion of GL was 271 ± 37, 95 ± 4, and 95 ± 11 ng/mL, respectively (Fig 3). Therefore, the histamine concentration in the GL-infused group was significantly decreased 2 and 7 days after infusion. Mean concentration of histamine 0 and 7 days after infusion was 197 ± 17 and 138 ± 23 ng/mL, respectively, in the antimicrobial-infused group. Although the histamine concentration decreased in the antimicrobial-infused group, the values did not decrease significantly.

Compared with the value for day 0, mean percentage inhibition of histamine production in milk was significantly (P < 0.01) less 2 and 7 days after infusion of GL (62.0 ± 4.5 and 62.8 ± 4.9%, respectively; Fig 3).

Moreover, the inhibitory effect of GL infusion on histamine production differed significantly from the corresponding value for the antimicrobial-infused group on days 2 and 7 after infusion.

Effects of GL on histamine production by milk leukocytes—Inhibitory effects of GL on histamine production by milk leukocytes were examined. Incubation of milk leukocytes for 24 hours in serum-free medium containing 1% human serum albumin and various concentrations of GL caused a concentration-dependent decrease in histamine content of the conditioned medium (Fig 4).
Discussion

Glycyrrhizin is an anti-inflammatory reagent. Thus, we investigated whether the infusion of GL promoted anti-inflammatory effects in mammary glands with mastitis caused by a low concentration of CNS in milk. Among the clinical signs, reductions in swelling and firmness of the mammary glands were observed during the period from 0 to 7 days after infusion of GL, whereas a reduction in the number of clots was promoted in both the GL- and antimicrobial-infused groups. Analysis of these results suggests that intramammary infusion of GL suppressed acute intramammary inflammation caused by CNS.

Intramammary infusion of an antimicrobial decreased mean bacterial content in the milk samples and may have decreased the concentration of bacterial toxins in the milk. However, in antimicrobial-treated glands, inhibition of histamine was weak, resulting in poor suppression of intramammary inflammation.

Although the bacterial concentration was not reduced in the GL-infused group, GL apparently exerted an anti-inflammatory effect, compared with the effects after infusion of the antimicrobial. Because CNS remained in the mammary tissue, it was suggested that GL had a moderate regulatory effect on the production of inflammatory mediators (eg, histamine), which was induced by bacteria, until at least 7 days after infusion.

In another study, investigators reported that some CNS were eliminated as a result of the milking procedure or during lactating periods. Therefore, CNS infection with a low bacterial concentration may be amenable to improvement by use of GL alone, compared with results for treatment of other virulent bacterial infections.

Regardless of the reduction in neutrophils after infusion of GL, we did not detect an increase in the bacterial concentration. Local accumulation of neutrophils is a physiologic response designed to eliminate invading pathogens. However, effects on the phagocytic and bacteriolytic activity of neutrophils are reduced in lactating cows because of casein and fat in milk. Also, prolonged exposure to a large number of neutrophils may result in destruction of mammary gland epithelial cells, causing a subsequent state of irreversible atrophy. The study reported here documented that intramammary infusion of GL reduced the migration of leukocytes, especially neutrophils, into affected mammary glands. Glycyrrhizin enhances CD4 T helper-1-type cytokine through the induction of IL-12, resulting in promotion of the cell-mediated immune response. Therefore, control of bacterial concentrations by the cell-mediated immune response may involve bacterial elimination without excessive neutrophils in mammary glands after infusion of GL alone.

Infusion of histamine into a mammary gland of a cow induces swelling and firmness in the gland as a result of enhancement of vascular expansion and permeability. Zia et al reported that the concentration of histamine in normal milk during lactation was low and increased from 44 to 300 ng/mL during intramammary infection. The method for quantification of histamine in the study reported here can be used to determine more precisely the concentration of histamine, compared with the method used by Zia et al, because many kinds of amines that interfere with the assay of histamine, such as putrescine and spermidine, are excluded from samples by passage through a phosphorylated column.

Because inhibition of histamine concentrations in milk was more effective in the GL-infused group than the antimicrobial-infused group, elimination of swelling and firmness by GL may be considered strong, compared with the results for infusion of the antimicrobial. Because GL reduced the production of histamine by milk leukocytes in culture, GL may reduce the concentration of histamine in milk through inhibition of the induction of histamine in inflamed mammary tissue. These findings suggested that intramammary infusion of GL decreased the concentration of histamine in milk, resulting in an improvement in the clinical condition of cows with mastitis.

Glycyrrhizin also inhibits prostaglandin E2 production by activated macrophages, and the optimal concentration of GL does not have a toxic effect on cells. Glycyrrhizin reportedly inhibits the enzymic activity of secretory type-II A phospholipase A2 (sPLA2-II A), which plays an important role in inflammation, because casein kinase II-mediated phosphorylation of sPLA2-II A is inhibited by GL and a glycyrrhetinic acid derivative. Analysis of those reports suggests that GL blocks the arachidonate cascades, resulting in inhibition of intramammary inflammation.

In contrast to results for the antimicrobial-infused group, mean SCC and mean percentage of neutrophils in the GL-infused group decreased, compared with the values prior to infusion. Increases in SCC in milk and migration of neutrophils into affected mammary
glands are induced by chemoattractants. It has been reported that GL inhibits the activation of complement by preventing the deposition of C2. Furthermore, investigators in another study reported that GL can block the arachidonate cascade. Analysis of those reports suggests that GL regulates the release of chemoattractants (eg, C5a and leukotriene B4) in inflamed tissues in cows, resulting in a decrease in migration of neutrophils from the blood into the mammary tissue.

Lactoferrin is released from specific granules of neutrophils and is also produced by inflamed mammary tissue. The concentration of lactoferrin in milk during lactating periods is low (< 450 µg/mL) but increases with the initiation of intramammary inflammation. Therefore, the concentration of lactoferrin is a marker of intramammary inflammation. In the study reported here, the decrease in the amount of lactoferrin in milk for the GL-infused group may have been closely related to the decrease in the percentage of neutrophils after intramammary infusion of GL. Although bovine neutrophils and lactoferrin are effective for eliminating staphylococci during nonlactating periods, prolonged exposure to a large number of neutrophils in milk may cause irreversible damage to the mammary glands, resulting in poor milk production during subsequent lactating periods.

The concentration of α-lactalbumin in milk from healthy mammary glands during lactation is approximately 1,100 µg/mL (range, 1,000 to 2,000 µg/mL) and it decreases during intramammary inflammation. Therefore, the concentration of α-lactalbumin is a marker of recovery in the mammary glands. In mammary glands with mastitis, the production of this protein may decrease because of damage to mammary epithelial cells caused by augmented intramammary neutrophils. Because the infusion of GL apparently increased the amount of α-lactalbumin in milk, production of α-lactalbumin may have increased as a result of improvement in the mammary tissue. These findings suggested that intramammary infusion of GL reduced intramammary inflammation, and the injured mammary gland recovered.

It is speculated that combined treatment with GL and an antimicrobial would improve the clinical condition and suppress bacteria; thus, a combined treatment of GL plus an antimicrobial may provide a high cure rate in practical applications. Additional studies are needed to investigate whether the curative effect on severe mastitis, that is caused by other bacteria (eg, S. aureus and Escherichia coli) after infusion of GL in combination with an antimicrobial is more effective than infusion of the antimicrobial alone.

Infusion of GL as treatment for cows with naturally occurring mastitis, which involves moderate inflammation caused by infection with CNS, protects the mammary glands of cows from excessive damage attributable to an excessive number of neutrophils. However, more investigations are necessary to clarify the mechanisms for the effects of GL on regulation of migration of neutrophils and production of histamine. In general, naturally acquired CNS infections are either treated with an antimicrobial or are left untreated, because the degree of inflammation is mild. However, intramammary infusion of GL soon reduces the inflammation, and GL may prevent persistent firmness of the affected mammary gland and a high SCC in milk. Analysis of these results suggested that intramammary infusion of GL early in CNS infection of mammary glands could be a safe method for inhibiting inflammation of mammary glands during lactation.

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