The Effect of Ascorbic Acid and \( \text{L}-\text{Histidine Therapy} \) on Acute Mammary Inflammation in Dairy Cattle

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ABSTRACT

Ascorbic acid and \( \text{L}-\text{histidine} \) were investigated as antioxidant therapies for acute mammary inflammation. Mastitis was induced in eight nonpregnant Holstein cows by intramammary infusion of endotoxin. Treatments were administered in a \( 4 \times 4 \) Latin square crossover design with 1-wk periods between challenges with endotoxin. Four individual treatments, control, ascorbic acid only, \( \text{L}-\text{histidine} \) only, and ascorbic acid plus \( \text{L}-\text{histidine} \), were applied. Two doses of 25 g of ascorbic acid administered intravenously at 3- and 5-h postendotoxin challenge increased milk production recovery (9% higher, \( P < 0.02 \)) and tended to reduce the extent of rumen stasis. Two doses of 25 g of \( \text{L}-\text{histidine} \) similarly administered decreased plasma antioxidant activities 5.5% (\( P < 0.05 \)). However, ascorbic acid and \( \text{L}-\text{histidine} \) had no effects on rectal temperature, heart rate, respiratory rate, and dry matter intake. The data suggested that ascorbic acid provided some potential benefit for recovery from acute mammary inflammation in dairy cattle.

(Key words: ascorbic acid, inflammation, \( \text{L}-\text{histidine} \), mastitis)

Abbreviation key: AA = ascorbic acid, AAPH = 2,2′-azobis (2-amidinopropane) hydrochloride, AOA = antioxidant activities, IgG1 = immunoglobulin G, \( \text{L}-\text{His} \) = \( \text{L}-\text{histidine} \), LPS = lipopolysaccharide, PSS = pyrogen-free physiological saline solution.

INTRODUCTION

Coliform organisms are the most common causes of severe mastitis among clinical cases. Hogan et al. (1989) found that coliforms accounted for 58.9% of severe clinical cases, and up to 29.2% of clinical coliform cases were classified as severe mastitis with abnormal milk, swelling of the quarter, and systemic signs (Eberhart, 1979). Antioxidant nutrition is an important part of coliform mastitis prevention because of the critical role of these micronutrients in mammary resistance to this disease (Bowers, 1997; Erskine, 1993; Smith, 1984). However, the therapeutic potential of antioxidants for the treatment of coliform mastitis has not been reported.

Micronutrients that are associated with antioxidant activity including vitamin A, vitamin E, \( \beta \)-carotene, selenium, zinc, and copper have been studied for their effect on mastitis (Bowers, 1997; Chew, 1996; Erskine, 1993). Results demonstrated that antioxidant supplementation could decrease the duration, incidence, and severity of clinical mastitis (Erskine et al., 1989; Smith et al., 1984) and was associated with lower prevalence of IMI caused by contagious pathogens (Erskine et al., 1987).

Ascorbic acid (AA) is a water-soluble, cytosolic, chain-breaking antioxidant (Machlin and Bendich, 1987) that is produced in the liver of cattle (Eicher-Pruiett et al., 1992; Itze, 1984). Hence, the biosynthetic capacity for AA in adult ruminants is believed to be sufficient for physiologic requirement (Itze, 1984). Nonetheless, ruminants can be prone to AA deficiency due to impaired synthesis, and rapid destruction by ruminal microflora (Itze, 1984). Dwenger and colleagues (1994) suggested that the scavenging of reactive oxygen metabolites is responsible for the AA-induced improvement in endotoxin-induced acute lung injury. In vitro chemiluminescence response following zymosan exposure was significantly higher in neutrophils collected from sheep treated with endotoxin alone than in neutrophils from sheep treated with endotoxin and therapeutic ascorbic acid (Dwenger et al., 1994). Therefore, generation of reactive oxygen metabolites and subsequent inflam-
mation could be less severe in AA-treated ruminants affected by endotoxin shock.

L-Histidine (L-His) is an essential amino acid (Chalupa and Sniffen, 1991; Peterson et al., 1998) and has been classified as an antioxidant (Kawamoto et al., 1997; Peterson et al., 1998). Maslinski et al. (1993) found that histamine concentrations in bovine milk were higher (317 ± 29 nmol/L) than that in bovine plasma (4.83 ± 0.82 nmol/L), suggesting both a need and a selective recruitment mechanism for this micronutrient in the bovine mammary gland. Histamine induces contraction of myoepithelial cells of alveoli and small ducts in mammary gland, that in turn stimulate milk secretion or milk ejection (Maslinski et al., 1993).

Evidence supports L-His as an extremely effective scavenger of •OH (Nagy and Floyd, 1984). In mice, L-His reduced intestinal membrane permeability in a model of experimentally induced bacterial diarrhea (Peterson et al., 1998). Kawamoto et al. (1997) also reported L-His protected against ischemia-reperfusion induced injury in the cerebrum of the rat.

Although studies demonstrating the potential benefits of AA and L-His in various laboratory animals are well recognized (Cimmins and Brunner, 1989; Eicher-Pruiett et al., 1992; Kawamoto et al., 1997; Maslinski et al., 1993; Peterson et al., 1998; Roth and Kaeberle, 1985), the potential therapeutic benefits of these antioxidants in dairy cows with coliform mastitis are unknown. The purpose of this study was to determine the effects of parenteral AA and L-His therapy on acute mammary inflammation and systemic indicators of clinical disease following acute endotoxin-induced mastitis in dairy cows.

MATERIALS AND METHODS

Animals

We selected eight nonpregnant Holstein cows that were near the end of lactation with clinically normal milk and mammary glands, i.e., quarter SCC less than 400,000 cells/ml and negative bacterial cultures at 48 h before endotoxin challenge. Cows were housed in tie stalls and fed a TMR balanced for a 40.9-kg milk production. Dry matter intakes were recorded each day. Cows were milked twice daily at approximately 10- to 12-h intervals by a quarter milking machine, from Monday evening through Saturday morning for each of the four treatment periods. All quarters were postdipped with 1% iodophor germicide after each milking. A jugular catheter was aseptically inserted at 12 h before endotoxin challenges and remained in each cow until the end of data collection. Data regarding age, milk production, day of lactation, and lactation number were also recorded.

Endotoxin-Induced Mastitis

Endotoxin solution (20 µg/ml) was prepared by dissolving 100 µg of a commercial Escherichia coli O111:B4 endotoxin (Sigma Chemical Co., St. Louis, MO) in 5 ml of pyrogen-free physiological saline solution (PSS, The Butler Company, Columbus, OH), which was then filtered by a 0.22-µm low extractable filter unit (Sterile D-GS, Millipore Industria E. Comerico Ltda., Brazil). The suspension was stored at 4°C and vigorously shaken before infusion. On Tuesday mornings, immediately after milking, the entire 100-µg preparation was intracisternally infused into 1 quarter/cow via syringe and 3.4-cm disposable J-12 teat infusion cannula (Jorgensen Laboratories, Inc., Loveland, CO). Before infusion, the teat was disinfected with alcohol. The infused teats and quarters were immediately massaged for 15 to 20 s in order to distribute the endotoxin solution.

Clinical Monitoring

Rectal temperature, ruminal contraction rate, heart rate, and respiratory rate were all measured at -12, 2, 3, 4, 6, 9, 12, 24, 36, and 48 h postchallenge. Quarter and milk appearance were also observed and compared with the appearance before LPS challenge.

Preparation of L-His and AA Solutions

The AA solution was prepared from AA injectable solution (The Butler Company) by diluting 25 g of AA into 500 ml of pyrogen-free PSS. The L-His (ICN Biomedicals Inc., Aurora, OH) solution was also prepared by dissolving 25 g of L-His in 500 ml of pyrogen-free PSS and dissolving with a stir bar on a warm magnetic stirrer for about 35 min. The solution was vortexed and then filtered with a 0.22-µm low extractable filter unit (Sterile D-GS, Millipore Industria E. Comerico Ltda.). Two 25-g doses (50 g total) of AA and L-His were slowly administered by intravenous injection via the jugular catheter. To mimic practical therapy of a clinical case of coliform mastitis, the initial dose was administered intravenously 3 h after endotoxin challenge, at which time clinical signs had appeared. We determined from the previous research that the clearance of 25-g dose of AA or L-His from plasma occurs within 2 h. Thus, we administered the second dose at 5 h after the endotoxin challenge.

Experimental Design

A 4 × 4 Latin-square crossover design was used for treatment assignment. Each Holstein cow was selected to complete each of the four treatments. The treatments included LPS challenge as control, LPS and AA, LPS
and L-His, and LPS, L-His and AA. The experiment was started by using the left-front quarter of the first cow for one of the four treatments. The left front quarter of the other cows was then randomly assigned to each of the other treatments by selecting cow numbers from a box and proceeding in order down a table. Each quarter was used one time for endotoxin-induced mastitis, thus all four quarters were eventually used over the four different periods from each cow, one treatment per quarter. Each period was 1 wk long.

**Milk Collection**

After aseptic preparation and discarding of foremilk, milk samples were collected to determine SCC, bacteriology, and immunoglobulin G1 (IgG1) concentration at 12 h before challenge, immediately before challenge, and 2, 3, 4, 6, 9, 12, 24, 36, 48, 60, 72, and 96 h and 1 wk after challenge. All milk samples were stored in crushed ice immediately after collection. One vial of milk preserved with a bronopol pellet was sent to the DHIA laboratory of Michigan for somatic cell counting and recorded as cells/ml. A second vial was collected for immediate bacteriological culture on 5% sheep blood agar plates, which were incubated at 37°C and initially observed at 24 h. If no microorganism was isolated, incubation continued until 48 h. A third sample was collected into a vial containing 0.05 ml of 1 M benzamidine HCl (a protease inhibitor, ICN Biomedicals Inc.) and centrifuged at 1000 x g for 15 min to separate cells and fat from skim milk. The whey was then prepared for IgG1 measurement by modifying the procedure of Guidry et al. (1980). Briefly, the skim layer beneath the fat was transferred to a new vial, and 5 µl of glacial acetic acid (Mallinckrodt U.S.P., Paris, KY) was added to precipitate casein. The solution was then centrifuged at 16,060 x g (Biofuge pico, Heraeus, NJ) for 13 min, and the remaining supernatant was decanted into a clean cryovial with 0.05 ml of 2 M KOH (Columbus Chemical Industries, Inc., Columbus, WI) and frozen at -20°C until IgG1 was determined. A commercial IgG1 single radial immunodiffusion kit (VMRD, Inc., Pullman, WA) was used to determine IgG1 concentration, recorded as mg/ml.

**Blood Collections for Determination of AA and L-His**

Blood samples were collected at 12 h before challenge, immediately before challenge, and 2, 3, 4, 6, 9, 12, 24, 36, 48, and 72 h after challenge. Blood samples were obtained by jugular catheters into heparinized Vacutainers and immediately placed in crushed ice. Sodium citrate was applied to catheters at each blood sampling to prevent coagulation in the catheter. Blood samples were centrifuged at 3000 x g (Dynac Centrifuge, Becton Dikenson, Sparks, MD), 4°C for 15 min to separate plasma. Duplicate 200- and 750-µl samples of plasma were pipetted into cryovials to determine AA (µM/L) and antioxidant capacities (% inhibition), respectively. Nitrogen was added to headspace and samples were stored at -80°C. Ascorbic acid analysis was performed at the Animal Health Diagnostic Laboratory, Nutrition Section, Michigan State University (East Lansing). Antioxidant capacities were analyzed at the laboratory of R. J. Harmon, University of Kentucky (Lexington).

**Ascorbic Acid (AA) Protocol**

Plasma AA was measured by HPLC using isocratic mobile-phase buffers (5% MeOH and 95% H2O with ion paring agent) and a reverse-phase, C18 column (Nova-Pack C18, Waters Corp., Milford, MA) coupled with ESA Coulouchem II electrochemical detector (Chelmsford, MA) and compared with a known AA standard (10 ng). Briefly, each sample (200 µl) was mixed with buffer (400 µl of 1 mM 90% methanol in water saturated with EDTA) to precipitate protein. They were vortexed, incubated on ice for 10 min, and centrifuged at 3000 rpm, 4°C for 15 min. The supernatants were then transferred to another set of plastic microtubes and placed on ice. Before running the samples, the column was prepared by passing decreasing concentrations of MeOH, then the mobile-phase buffer though the entire system under the approximate 2000-psi pressure. The pump seal was rinsed daily with 10% MeOH. Ten-microliter samples were injected into the prepared HPLC column, and quantified by single-point calibration using area integration and known AA standard (10 ng).

All chromatographic calculations were done automatically using the Millenium chromatography software (Waters Corp.). External standards were used in the quantification and sample concentration determined by the specific peak of each sample and 10 ng of AA, respectively. Both inter- and intraassay coefficient of variations, which were 13.6 and 5.9%, respectively, were also included in the calculations. The content for each sample was divided by injection volume (10 µl), multiplied by a dilution factor (3, the addition of tissue buffer), and finally reported as concentrations of AA in plasma (µM/L).

**Antioxidant Activities (AOA) Protocol**

Antioxidant activity of plasma, which inhibited chemical damage to phycoerythin induced by the oxidative agent, 40 µM 2,2′-azobis (2-amidinopropane) hydrochloride (AAPH), was detected by the rate of decay phycoer-
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Ythin fluorescence emission as described by Glazer (1988). For the control, 3.58 ml of 75 μM sodium phosphate buffer (pH 7.0), 0.02 ml of 1.7 × 10⁻⁶ M B-phycoerythrin, and 0.4 ml of AAPH were mixed at 37°C. Into each sample tube, 0.2 ml of diluted plasma (1:320 dilution in the final volume) was added in the same mixtures in place of 0.2 ml of buffer. The solution was excited at 525 nm, and the emission was read at 575 nm. The fluorescence was measured at 37°C in a digital fluorometer immediately before and at 5-min intervals for 40 min after the addition of AAPH. Sample AOA was calculated and reported as percentage inhibition values of the decay of fluorescence (FL) of the compound phycoerythrin. The percentage inhibition was calculated as:

\[ \% = \left( \frac{\text{change FL control} - \text{change FL sample}}{\text{change FL control}} \right) \times 100. \]

The change in FL was that which occurred over the 40-min incubation. Hydroxyl radicals are generated by this reaction and cause the decay. The greater the percentage inhibition, the greater the antioxidant capacity of the plasma. The control in the assay was the rate of decay of fluorescence with no antioxidant plasma present. The samples were measured in duplicate and the means were calculated.

Statistical Analysis

A repeated measures analysis (Statistical Analysis System, SAS Institute 1989–1996) was used for comparisons among the four treatments. Specific contrasts were used to determine the effects of AA, L-His, non-AA, and non-L-His group. Period (wk 1, 2, 3, and 4), front or hind quarter, and cow were also included as independent variables. The test for sphericity on the GLM printout (Mauchly’s criterion) applied to orthogonal components was used to indicate whether a multivariate analysis was needed. With this statistical method, the following dependent variables were tested: milk production, rectal temperature, log10 SCC, milk IgG1, AOA, heart rate, respiratory rate, and ruminal contraction rate. A period variable was included to adjust for carryover effects of the previous endotoxin treatments. A sufficiently long time was allowed between the administration of different treatments to the same cow. Due to a small sample size (eight cows) with many repeated measures, comparisons between treatments were combined into three to four groups of sample period in the order of the time.

RESULTS

The data were analyzed to compare responses of AA-treated cows with responses of cows that were not administered AA, and to compare responses of cows treated with L-His with those not treated with L-His. Comparisons among individual treatment groups (control, AA only, L-His only, and AA plus L-His) were made to present a concise discussion on the critical hypothesis of this research. However, we determined that there was no statistical significance of the interaction between AA and L-His in all variables (Table 1).

Milk Production

Milk from nonchallenged quarters remained normal in appearance and bacteriologically negative throughout the period of study. There was no significant difference between AA and non-AA treatments, as well as between L-His and non-L-His treatments on quarter milk production throughout the trial (data not shown). However, daily composite milk production following AA treatments was significantly higher (P < 0.02) from d 3 to 5 as compared to the milk production in the non-AA treatments (Figure 1). There was no significant difference for mean composite milk production in non-L-

Table 1. Repeated measures analysis regarding the interaction between amino acid and L-histidine treatments (AA*L-His) on each variable reported as P value.

<table>
<thead>
<tr>
<th>Variables</th>
<th>A A*L-His (P-value)</th>
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</thead>
<tbody>
<tr>
<td>Milk production</td>
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<tr>
<td>Antioxidant activity</td>
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<tr>
<td>Rectal temperature</td>
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<tr>
<td>Ruminal contraction rate</td>
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<td>Respiratory rate</td>
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<td>Heart rate</td>
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<td>Somatic cell count</td>
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<tr>
<td>Milk IgG1</td>
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</tr>
<tr>
<td>DMI</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Figure 1. Effect of ascorbic acid (AA) on daily composite milk production in dairy cattle with endotoxin-induced mastitis. Daily composite milk production in AA cows (●) was significantly higher than in non-AA cows (□); **P < 0.05. Arrow indicates the day of lipopolysaccharide (LPS) and AA infusion.
His treatments compared with L-His treatments at any milking time (data not shown).

**Milk IgG1**

IgG1 concentration (mg/ml) of milk following AA treatment tended to be higher than in non-AA cows at 6 and 24 h after LPS challenge ($P < 0.10$, Figure 2). Mean milk IgG1 concentrations following L-His treatment peaked at 12 h (1.82 ± 0.2 mg/ml), and were significantly lower ($P = 0.0555$) than in non-L-His treatments from 3 to 9 h post-LPS challenge (Figure 3).

**Antioxidant Activities (AOA)**

Mean antioxidant activities (percent inhibition) following L-His treatment were lower than non-L-His treatment from 6 to 12 h post-LPS challenge ($P < 0.05$; Figure 4). There was no significant difference between AA and non-AA treatment at any time after LPS challenge (Figure 5).

**Plasma Ascorbic Acid**

Mean AA concentration ($\mu$M/L) rapidly increased after the first infusion, peaked after the second infusion at 6 h post-LPS challenge (approximately 650 $\mu$M/L), and rapidly dropped afterward (Figure 6).

**Somatic Cell Count**

Somatic cell count for all challenges rapidly increased by 2 to 3 h after infusion, and reached a peak of greater than $1 \times 10^7$ cells/ml by 9 to 12 h after infusion (data not shown).
Neither AA nor L-His affected mean rectal temperature after LPS challenge. However, mean rectal temperature in period 1 (first endotoxin challenge for each cow) was significantly lower than in periods 2 and 3 (second and third endotoxin challenge for each cow) from 0 to 4 h post-LPS challenge ($P < 0.05$, Figure 7). Mean rectal temperature in period 4 was also significantly lower than in periods 2 and 3 from 3 to 4 h post-LPS challenge ($P < 0.05$).

**Heart and Respiratory Rate**

Neither heart rate nor respiratory rate was affected by AA or L-His throughout the experimental period (data not shown).

**Ruminal Contraction Rate**

Mean ruminal contraction rate following AA treatment tended to be higher ($P < 0.10$) than in non-AA challenges (Figure 8). However, there was no significant difference between L-His and non-L-His cows at any time.

**Dry Matter Intake**

Mean percentage change of DMI in all treatments markedly decreased in post-LPS challenge. Compared with 1 d before LPS challenge, there were no significant differences between AA and non-AA treatments, or between L-His and non-L-His treatments (data not shown).

**DISCUSSION**

The Latin square crossover design allowed for use of fewer experimental subjects while expanding the number of observations. However, there are two important drawbacks with this design: carryover effects and washout periods (Fleiss, 1986; Zar, 1996). The effect of the treatment given in one period might carry over into the next period and thus obscure the effect of subsequent treatments if a sufficient time (a washout period) was not allowed between the administration of different treatments (Fleiss, 1986; Zar, 1996). However, this potential problem was not likely in our study because of the reversible effect of endotoxin on the mammary gland parenchyma (Schalm, 1977) and a self-limited response (Shuster and Harmon, 1991). In addition, both AA and L-His have short half-lives (Roth and Kaeberle, 1985; Sitton et al., 1988), particularly AA, for which the serum concentration peaked and decreased to the pretreatment levels within hours after infusion (Figure 6).

In this study, rectal temperature increased after intramammary administration of endotoxin. This has...
been observed in previous studies (Lohuis et al., 1988; Shuster et al., 1993). Rectal temperature was not affected by AA or L-His, and cows fully responded to subsequent LPS challenges with predictable rises of rectal temperature (Figure 7). However, there was a modest effect of period on rectal temperature, which may have been related to environmental temperature in the barn.

Previous studies demonstrated the tolerance to E. coli endotoxin induced by repetitive daily intravenous (Lohuis et al., 1988) or intramammary administration (Shuster and Harmon, 1991). Cows infused with 10 µg of E. coli endotoxin in the same two homolateral quarters twice daily for several days became partially refractory to subsequent infusions in terms of systemic, but not local, effects (Shuster and Harmon, 1991). This phenomenon was not observed in the present study, which was conducted by endotoxin infusion at weekly intervals. Milk production declined markedly following endotoxin infusion, but recovered completely before the beginning of each consecutive treatment. It is possible that in the previous study the dose of 10 µg of intramammary endotoxin infusion was too small a dose to induce enough inflammatory mediators for a systemic response. Generally, it only causes a mild to moderate mastitis and systemic response (Shuster and Harmon, 1991; Shuster et al., 1993).

Pathological changes in milk result from an increased capillary permeability with an outflow of plasma proteins such as serum albumin and IgG (Kitchen, 1981; Schalm, 1997). In this study, we used IgG1 as an indicator of a mammary inflammation rather than serum albumin. Additionally, we believe milk concentration of IgG1 provides a more accurate measure of inflammation than SCC in an acute mastitis model. Because of the severe abnormality and the presence of many flakes and clots in the milk shortly after treatments, SCC were highly variable. L-His-treated cows had lower IgG1, signifying decreased inflammation, which was unexpected because L-His is a precursor of histamine a proinflammatory vasoactive amine (Maslinski et al., 1993). Although IgG1 is generally correlated with inflammation, this experimental model suggests it may not be a good indicator of milk production recovery as evidenced by increasing time to milk recovery for L-His-treated cows in our study.

Although reports support L-His as an antioxidant in vitro and in disease models (Kawamoto et al., 1997; Nagy and Floyd, 1984; Peterson et al., 1998), in our study L-His decreased plasma AOA. Studies have demonstrated that L-His triggered in vitro H2O2-mediated cellular (particularly DNA) damage and cytotoxicity in mammalian cells (Cantoni et al., 1992; Guidarelli et al., 1995; Tachon et al., 1994). In an environment with concomitant H2O2 presence, parenteral L-His administration may not function as an antioxidant but rather as an oxidative catalyst. Our data suggest that L-His was antagonistic to this antioxidant activity (Figure 4).

In contrast, parenteral AA administration did not affect plasma AOA in this study. However, a more rapid return in milk production was observed following AA treatment, compared with non-AA treatment, thus, plasma AOA may not be the best indicator of AA influence on antioxidant status.

Experimental endotoxin-induced mastitis or field cases of E. coli mastitis have not been associated with reduced rumen motility in some studies (Lohuis et al., 1988; Verheijden et al., 1983). We observed decreased rumen motility in this study, although the change in frequency was <1 contraction/min and may not be biologically significant. This is in agreement with recent studies of clinical mastitis caused by gram-negative bacteria (Lohuis et al., 1990; Morin et al., 1998). Ascorbic acid treatment tended to reduce the extent of this clinical sign in the current study.

In summary, cows treated with AA therapy following endotoxin-induced mastitis had increased the rate of milk production recovery and a quicker recovery from rumen stasis. On the other hand, L-His decreased the antioxidant activities in plasma. Most of the other outcomes measured were prone to high variation. The results of this study are encouraging and support further research on AA treatment of acute coliform mastitis. The effect of AA on field cases of coliform mastitis might be even greater than observed in this study because experimental endotoxin challenge may not cause a sufficiently severe or complex clinical mastitis to attain differences in outcome variables between treatments as compared to natural E. coli challenges. Further research is necessary to fully understand and elucidate the potential roles of AA and L-His on acute mammary inflammation and systemic shock caused by coliform bacteria in dairy cattle.

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