Short Communication: Influence of *Staphylococcus aureus* Intramammary Infection on Serum Copper, Zinc, and Iron Concentrations

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ABSTRACT

The goal of the present study was to characterize changes in serum trace mineral concentrations in cattle with experimentally induced *Staphylococcus aureus* mastitis. Nine primiparous Holstein-Friesian cattle were challenged with approximately 150 cfu of *Staph. aureus* ATCC29740 by intramammary infusion on d 6, 7, and 8 of lactation. Serum Cu, Zn, and Fe concentrations were determined immediately before and at 24, 48, and 72 h after the final intramammary infusion of *Staph. aureus*. Infection status (cfu/mL of *Staph. aureus*), milk somatic cell count, and mastitis score were also determined at these times. Infection resulted in a decrease in mean serum Cu, Zn, and Fe concentrations to 89, 83, and 81% of preinfection concentrations at 24 h postchallenge. One-way analysis of variance for repeated measures demonstrated a significant change in serum zinc concentration. The reductions in trace mineral concentrations were of less magnitude than observed following experimental *E. coli* mastitis.

(Key words: *Staphylococcus aureus*, mastitis, copper, zinc, iron)

Alterations in plasma Cu, Zn, and Fe concentrations have been documented in experimental coliform mastitis models (Erskine and Bartlett, 1993; Lohuis et al., 1988a, 1988b, 1990). Lohuis and co-workers (1988b) demonstrated a dramatic decline in plasma Zn and Fe concentrations following intramammary infusion of 2×10^6 cfu of *Escherichia coli*. Similarly, Erskine and Bartlett (1993) showed that intramammary infusion of a minimum infective dose (50 cfu) of *E. coli* resulted in a decline in mean serum concentrations of Zn, Fe, and Cu to 28, 35, and 52% of prechallenge concentrations,

respectively. The decline in mean serum Cu concentration was not significant ($P > 0.05$).

Reductions in serum Zn and Fe are regarded as non-specific host defense mechanisms against bacterial infection. Lohuis and co-workers (1990) demonstrated that the numbers of *E. coli* shed from inoculated mammary quarters were positively correlated with plasma Zn and Fe concentrations. Iron withholding during microbial infection is regarded as host protective and is mediated by a number of host mechanisms, including decreased intestinal absorption of Fe, decreased release of Fe from macrophages, increased Fe storage through increased synthesis of ferritin, release of lactoferrin (an iron-binding glycoprotein) from neutrophils and epithelial cells, and, in some species, increased urinary Fe excretion (Weinberg, 1984). Microbial metabolism requires trace concentrations of Zn (Sugarman, 1983). In hamsters, a decrease in serum Zn concentrations following intraperitoneal administration of endotoxin has been attributed to increased uptake by the liver (Etzel et al., 1982). In addition, increases in metallothionein (a Zn-binding protein) have been documented during acute bacterial infection (Sobocinski et al., 1978). However, the complete role of Zn and its interaction with Fe as a nonspecific host defense against bacterial infection remain to be understood fully. Alterations in serum Cu concentrations have been recorded in several species using endotoxin or gram-negative bacterial challenge models (Etzel et al., 1982; Barber et al., 1988; Erskine and Bartlett, 1993). In rats and hamsters, a rise in serum Cu concentration was observed following parenteral administration of endotoxin. The increase in serum Cu concentration was attributed to increased synthesis of ceruloplasmin by the liver. In contrast to the rodent experiments, Erskine and Bartlett (1993) documented a decrease in plasma Cu concentrations following experimental IMI with *E. coli*. They attributed this difference to the fact that their experimental subjects were marginally Cu deficient at the beginning of their study. Previous work in rats had shown that Cu-deficient rats did not exhibit increased serum ceru-

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loplasmin concentrations following injection of interleukin-1, an inflammatory mediator thought to increase ceruloplasmin synthesis following endotoxin release (Barber et al., 1988).

Staphylococcus aureus is a contagious mastitis pathogen of dairy cattle that generally causes chronic, subclinical mastitis. The inflammatory response following *Staph. aureus* IMI is expected to be minimal in contrast to the profound inflammatory response described for acute coliform mastitis. Hence, the alterations in serum Cu, Zn, and Fe associated with acute coliform mastitis are not likely to occur in an experimental subclinical *Staph. aureus* mastitis model. No data could be found regarding alterations in serum Cu, Zn, and Fe concentrations following experimental *Staph. aureus* IMI. The objective of the present study was to quantify serum Cu, Zn, and Fe concentrations before and after experimental *Staph. aureus* IMI. The hypothesis was that *Staph. aureus* IMI would not cause significant alterations in serum Cu, Zn, and Fe concentrations.

The study population consisted of 12 healthy primiparous Holstein-Friesian dairy cattle. Cattle were fed a TMR balanced for stage of lactation and had free-choice access to water. Cattle were milked twice daily in a double-three parabone parlor and housed in a free-stall facility on sand bedding. The experimental protocol was approved by the University of Missouri institutional animal care and use committee.

Prechallenge IMI status was determined by culturing mammary quarter foremilk samples aseptically collected on d 1 to 5 of lactation. All cattle free of *Staph. aureus* IMI and free of other major mastitis pathogens ($n = 9$) were included in the trial. Cattle were infused with *Staph. aureus* (ATCC 29740, American Type Culture Collection, Rockville, MD) in a single infection-free mammary quarter on d 6, 7, and 8 of lactation. We chose to challenge with *Staph. aureus* on 3 consecutive days in order to ensure all heifers developed an IMI. Previous experience with experimentally infecting cattle with *Staph. aureus* suggested that this would give the best chance of getting all cattle infected (Middleton, unpublished data). Geometric mean colony-forming units of *Staph. aureus* infused on d 6, 7, and 8 of lactation were 154, 164, and 193, respectively. There were no significant differences in inoculum size between cows or day of infusion ($P = 0.34$ and $P = 0.41$, respectively).

Mammary quarter foremilk samples were aseptically collected immediately prior to experimental challenge (time 0) and at 24, 48, and 96 h postchallenge from all lactating quarters for milk culture and SCC determinations. Time 0 corresponds to d 6 of lactation and 24, 48, and 96 h postchallenge correspond to d 9, 10, and 12 of lactation, respectively. All milk samples for bacterial cultivation were stored at -20°C until further analysis.

Minimum storage time prior to culture was 24 h. All milk samples for SCC determination were mixed with a proprietary preservative (Mid-South Dairy Records, Springfield, MO) and stored at 6°C . Somatic cell count samples were shipped to an external laboratory (Mid-South Dairy Records, Springfield, MO) every few days for analysis. Mammary glands and milk secretions were scored once daily on d 1 to 12 of lactation for evidence of mastitis. Scoring criteria were: 0 = no overt changes in gland or milk, 1 = overt changes in milk with no observed mammary gland inflammation, 2 = local inflammation of the mammary gland accompanied by overt changes in milk, and 3 = severe clinical mastitis with systemic symptoms. Whole blood (10 mL) samples were collected into evacuated glass tubes with inert rubber stoppers (Royal Blue Top Vacutainer Tubes, Becton Dickinson, Franklin Lakes, NJ) via coccygeal venipuncture immediately prior to challenge (time 0) and at 24, 48, and 96 h postchallenge to determine serum Cu, Zn, and Fe concentrations. Blood was allowed to clot, and serum was harvested by centrifugation ($1000 \times g$, 15 min) and stored at -80°C until analyzed.

All milk samples for bacterial cultivation were thawed at room temperature and cultured according to established guidelines (Hogan et al., 1999). Briefly, approximately 0.03 mL of milk was spread evenly across half a Columbia blood agar plate with 5% sheep blood (Remel, Lenexa, KS) using a sterile, cotton-tipped applicator. Plates were incubated at 37°C for 24 h and at room temperature for a further 24 h. Plates were read at 24 and 48 h. Bacterial isolates were initially identified based on colonial morphology, hemolytic patterns, KOH gelation, and catalase reaction (Hogan et al., 1999). All suspect staphylococci were subjected to a coagulase test (Hogan et al., 1999). All coagulase-positive isolates were speciated using a commercial biochemical typing system (api STAPH, bioMerieux, Hazelwood, MO). *Staphylococcus aureus* bacteria counts (cfu/mL) were determined for the challenged mammary quarter immediately prior to infusion (time 0) and at 24, 48, and 96 h postchallenge by counting the number of colonies on the blood agar plate and dividing by 0.03. If the number of colonies were too numerous to count, serial 10-fold dilutions of milk were made in PBS solution (Dulbecco's PBS, Gibco, Grand Island, NY) and the appropriate calculations made to arrive at cfu/mL of *Staph. aureus*. All suspect streptococci were further differentiated using CAMP/aesculin media (Remel, Lenexa, KS). Suspected gram-negative (KOH gelation positive) isolates were not further differentiated.

Milk SCC determinations were performed by the regional Dairy Herd Improvement Association laboratory (Mid-South Dairy Records) using an electronic counter. Serum Cu, Zn, and Fe concentrations were determined

Figure 1. Mean linear score SCS and mean log10 cfu/mL of *Staphylococcus aureus* in the challenged mammary quarter over time. Time 0 represents mean prechallenge linear score cell count.

at the Veterinary Medical Diagnostic Laboratory (University of Missouri, Columbia) by atomic absorption spectrophotometry according to established guidelines (Osheim, 1983; Osheim and Ross, 1985).

Means and standard deviations were calculated for serum Cu, Zn, and Fe concentrations immediately prechallenge (time 0) and at 24, 48, and 96 h postchallenge. Similarly, means and standard deviations were calculated for SCC (as linear scores) and *Staph. aureus* bacterial counts (as log10 cfu/mL). Data were analyzed using a one-way analysis of variance (ANOVA) for repeated measures (NCSS 97, Number Cruncher Statistical Software, Kaysville, UT). Dependent variables were serum Cu, Zn, and Fe concentrations (ppm), linear score SCC, and *Staph. aureus* count (log10 cfu/mL). Independent variables were cow (random variable), time (fixed variable), and error. Results were considered significant at $P < 0.05$. When significant differences were detected with one-way ANOVA for repeated measures, Duncan's multiple-comparison test was used to determine which time periods differed from each other (NCSS 97, Number Cruncher Statistical Software, Kaysville, UT).

All challenged mammary quarters became infected with *Staph. aureus* and maintained their infections throughout the follow-up period. Three of the nine enrolled cattle developed clinical *Staph. aureus* mastitis (mastitis score 2) in the challenged mammary quarter, which persisted until 96 h postchallenge. All other cattle had subclinical *Staph. aureus* mastitis in the challenged mammary quarter. The median mastitis score for all challenged mammary quarters was 0. Mean linear score SCC and *Staph. aureus* bacterial counts (log10 cfu/mL) in the challenged mammary quarter over time are shown in Figure 1. Linear scores in the *Staph.*

Figure 2. Mean serum Zn concentration over time. Time points with different letters were significantly different from each other ($P = 0.02$). Error bars represent $\pm$ one standard deviation. Time 0 represents mean prechallenge serum Zn concentration.

aureus-infected quarter were significantly greater than prechallenge levels at 24 and 48 h postchallenge ($P = 0.0002$). Linear scores were not significantly different from prechallenge levels in any of the unchallenged mammary quarters at 24, 48, and 96 h postchallenge ($P > 0.06$). *Staphylococcus aureus* bacterial concentrations (log10 cfu/mL) in the challenged mammary quarter were significantly ($P < 0.01$) elevated following challenge but did not differ at 24, 48, and 96 h postchallenge, and there was no significant difference in the number of *Staph. aureus* (log10 cfu/mL) shed from the infected quarter between cows postchallenge ($P = 0.62$).

Means and standard deviations for serum Cu concentrations at time 0 (prechallenge) and 24, 48, and 96 h postchallenge were 0.89 ± 0.15 , 0.84 ± 0.11 , 0.88 ± 0.10 , and 0.89 ± 0.16 ppm, respectively. There were no significant differences in serum Cu concentrations over time ($P = 0.39$). In contrast to the relatively constant serum Cu concentrations, serum Fe and Zn concentrations declined immediately postchallenge and rebounded by 96 h postchallenge. Means and standard deviations for serum Fe concentrations at time 0 and 24, 48, and 96 h postchallenge were 1.70 ± 0.67 , 1.30 ± 0.65 , 1.23 ± 0.38 , and 1.66 ± 0.98 ppm, respectively. The decline in serum Fe concentration was not significant ($P = 0.22$). Means and standard deviations for serum Zn concentrations at each time point are shown graphically in Figure 2. The decline in serum Zn concentration was significant at 24 h postchallenge ($P = 0.02$).

To our knowledge, alterations in serum Cu, Zn, and Fe have not been previously characterized for *Staph. aureus* mastitis. The alterations in serum Cu, Zn, and Fe concentrations in the *Staph. aureus* mastitis model

described here are similar to those described previously in experimental coliform mastitis models in that we observed a decline in serum Cu, Zn, and Fe concentrations 24 h after the final *Staph. aureus* infusion. However, only the decrease in serum Zn concentration at 24 h postchallenge was significant ($P = 0.02$). The declines in trace mineral concentrations coincided with a rise in linear score SCC, suggesting that the declines were associated with mammary gland inflammation. Similarly, the rise in linear score SCC was associated with the rise in *Staph. aureus* cfu/mL shed in milk, suggesting that the mammary inflammation was caused by the *Staph. aureus* IMI.

Decreases in serum trace mineral concentrations in response to bacterial infection are thought to act as a nonspecific host defense mechanism sequestering microminerals such as Fe and Zn, which are vital for normal bacterial growth (Lohuis et al., 1988a). The exact physiology of these trace mineral alterations is not clearly understood, but it is believed that these trace mineral fluxes are related to increased concentrations of acute phase proteins, metal ion chelators, and alterations in host metabolism. Previously, Erskine and Bartlett (1993) showed a decrease in serum Cu, Zn, and Fe concentrations to 52, 28, and 35% of prechallenge concentrations at approximately 24 h post-*E. coli* challenge in a coliform mastitis model, whereas in the *Staph. aureus* mastitis model described here, mean serum Cu, Zn, and Fe concentrations only declined to 89, 83, and 81% of prechallenge concentrations at 24 h postchallenge, respectively.

We can only hypothesize as to why the fluctuations in serum trace-mineral concentrations in our *Staph. aureus* mastitis model were different from those previously described for *E. coli* mastitis, because no prior studies have evaluated serum trace-mineral fluctuations associated with *Staph. aureus* mastitis, and we did not correlate the alterations in Cu, Zn, and Fe with other indicators of inflammation. Several possibilities exist for the less significant alterations in serum Cu, Zn, and Fe concentrations in our *Staph. aureus* mastitis model versus those previously reported in *E. coli* mastitis models: (1) the inflammatory response following *Staph. aureus* intramammary infection is localized to the mammary tissue and released inflammatory mediators have little systemic influence and, therefore, little effect on serum trace mineral concentrations, (2) the more pronounced declines in serum Cu, Zn, and Fe concentrations reported in coliform mastitis models occur as a function of endotoxin release that stimulates the release of circulating endogenous mediators that cause the trace-mineral alterations, and/or (3) serum

Cu and Fe are less sensitive indicators of inflammation than serum Zn and, therefore, the milder inflammatory changes noted in *Staph. aureus* mastitis only influence changes in serum Zn.

In conclusion, the role of serum trace mineral sequestration as a nonspecific host defense against *Staph. aureus* mastitis is probably of less importance than that described for gram-negative infection. Further research correlating trace-mineral changes in both milk and serum with inflammation-induced factors, such as lactoferrin, metallothionein, and ceruloplasmin, would help better characterize the role of Cu, Zn, and Fe in *Staph. aureus* mastitis.

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