Short Communication: Changes in Micromineral, Magnesium, Cytokine, and Cortisol Concentrations in Blood of Dairy Goats Following Intramammary Inoculation with *Staphylococcus aureus*¹

J. L. Ma,* J. F. Wang,*² K. Wang,* C. X. Wu,* T. Lai,* and Y. H. Zhu†

*College of Veterinary Medicine, China Agricultural University, Beijing, China

†Department of Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden

ABSTRACT

The aim of this study was to investigate mineral metabolism and immune response in dairy goats following intramammary inoculation with varying doses of *Staphylococcus aureus*. Blood samples were collected at 0, 2, 4, 8, 12, 24, 48, and 72 h after intramammary inoculation. Lowered plasma Fe concentrations were observed from 12 to 24 h postinoculation in groups SAA (*Staph. aureus* at 10^4 cfu, $n = 5$) and SAB (*Staph. aureus* at 10^8 cfu, $n = 5$). Plasma Cu concentrations increased in group SAB 2 h after inoculation and maintained greater concentrations until the end of the experiment compared with the control group (phosphate-buffered saline, $n = 5$). Increased plasma Zn concentrations in group SAB were observed 48 h after inoculation, and the concentration was still greater 72 h after inoculation compared with the control group. Greater plasma Mg concentrations were detected in groups SAA and SAB compared with the control group at all timepoints after inoculation. Plasma Mg concentrations were generally greater in group SAA than in group SAB through 72 h (except at 2 h). Plasma tumor necrosis factor- α concentrations were unchanged following intramammary inoculation with *Staph. aureus* throughout the study. Plasma IL-6 concentrations in groups SAA and SAB increased gradually compared with the control group and peaked at 48 h after inoculation. In group SAB, serum cortisol concentrations started to increase from 8 h postinoculation and peaked at 12 h postinoculation. In conclusion, increasing the inoculum dose does not induce more rapid proinflammatory cytokine responses, whereas the data indicate that mineral metabolic alterations occur during the course of *Staph. aureus* mastitis in the goat.

Key words: mineral, cytokine, dairy goat, *Staphylococcus aureus*

Mastitis is one of the most costly diseases in dairy animals. It is an inflammation of the mammary gland that often develops in response to intramammary bacterial infections. *Staphylococcus aureus* is one of the most common etiological agents; it can provoke clinical mastitis but more frequently causes subclinical infections that tend to become chronic mastitis (Sears and McCarthy, 2003). The neuroendocrine system, immune system, and mineral metabolism interact to coordinate physiological responses to infection and inflammation. These directional adaptive responses include the induction of proinflammatory mediators, changes in mineral metabolism, fever, reduced feed intake, reduced milk production, diminished growth performance, and the development of a regulated specific immune response to ward off the pathogen. The reductions in serum Fe or Zn are regarded as nonspecific host defense mechanisms against bacterial infections that will decrease the availability of these divalent cations needed for bacterial growth (Failla, 2003). Following *Escherichia coli* or LPS infections, a decrease in the concentrations of Fe, Cu, Zn, Ca, and P in blood was observed in dairy cows, dairy goats, and lactating sows (Erskine and Bartlett, 1993; Wang et al., 2006, 2007). A number of reports have shown an increase in serum and plasma cortisol concentrations during inflammation and infection (Weiss et al., 1995; Zhu et al., 2004). So far, little has been reported about the changes in plasma Fe, Cu, Zn, and Mg concentrations during *Staph. aureus* mastitis. Few studies have focused on the influence of immune activation on mineral metabolism in dairy goats. Thus, the main objectives of this study were to investigate mineral metabolism and the immune response in dairy goats following intramammary inoculation with varying doses of *Staph. aureus*.

Fifteen dairy goats (Swiss Saanen) weighing 35 to 45 kg were obtained from a commercial farm in Hebei Province, China. The average age of the goats was 3 yr

Received December 21, 2006.

Accepted July 6, 2007.

¹This project was supported by the National Nature Science Foundation of China (project No. 30571363), and the China Agricultural University.

²Corresponding author: jiufeng_wang@hotmail.com

(2 to 4 yr) and they were 60 to 80 d postkidding. All goats were clinically healthy. Milk from each quarter was bacteriologically negative and had milk SCC <150,000 cells/mL measured by a Fossomatic cell counter (Fossomatic model 90; Foss Food Technology, Hillerød, Denmark). The goats were housed in stanchion barns and fed ad libitum with good quality hay and water. Concentrate was given twice daily. The goats were milked twice daily at 0730 and 1630 h, and their daily milk yield exceeded 2.0 L throughout the experiment. The experiment was carried out at the animal experimental facility of the College of Veterinary Medicine, China Agricultural University. The experimental protocol was approved by the China Laboratory Animal Care and Use Committee.

Staphylococcus aureus strain C56010 (China Veterinary Microorganism Preservation Center, Beijing, China) was originally obtained from a case of acute clinical dairy goat mastitis. The inoculum was prepared by growing the bacteria on ram blood agar medium (Sladek et al., 2005). Three colonies of this culture were inoculated into brain heart infusion broth and cultivated under continual rotation (30 rotations/min) for 18 h at 37°C. The stock culture was stored at 4°C until used. On the day of inoculation, 1 mL of the stock culture was inoculated into 5 mL of fresh brain heart infusion broth and incubated under continual rotation (30 rotations/min) for 4 h. Bacteria in the exponential phase of growth were harvested and washed once in pyrogen-free PBS. Total bacterial cell counts were determined using a hemocytometer, and the bacterial suspension was adjusted to achieve concentrations of 10^4 and 10^8 bacterial cells/mL. Each inoculum was tested by measurement of the bacterial count (cfu/mL) after 24 h of incubation at 37°C on ram blood agar medium.

The goats were randomly assigned to 3 groups. Two hours after the morning milking (0930 h), the goats in the control group ($n = 5$) were administered an intramammary inoculation with 1 mL of PBS, whereas the treatment goats were inoculated with 10^4 cfu (group A, **SAA**; 10^4 cfu, $n = 5$) or 10^8 cfu (group B, **SAB**; 10^8 cfu, $n = 5$) of *Staph. aureus*. The teat at the right side of the mammary gland was carefully washed with soap and disinfected with ethanol. A blunt needle was introduced through the teat canal on the right-side teat, and the inoculum (1 mL) was infused into mammary gland. Blood samples were collected via jugular venipuncture into glass tubes, at 0 (immediately before inoculation), 2, 4, 8, 12, 24, 48, and 72 h after inoculation with PBS or *Staph. aureus*. Rectal temperature was measured on all sampling occasions. Clinical examinations were performed twice daily throughout the experiment, and included palpation of mammary glands, measurement of rectal temperature, habitus, and appetite. In addition,

milk samples were collected twice daily before and after infection, and analyzed fresh for bacteriological growth.

Plasma Fe, Cu, Zn, and Mg concentrations were determined using atomic absorption spectrometry (Perkin-Elmer Corp., Norwalk, CT). Plasma TNF- α concentrations were determined using a commercially available ELISA kit (Bionewtrans Pharmaceutical Biotechnology Co. Ltd., Franklin, MA) specific for goat TNF- α with a minimum detectable concentration of 1.0 pmol/L. Plasma IL-6 concentrations were determined using a commercially available ELISA kit (Bionewtrans Pharmaceutical Biotechnology Co. Ltd.) specific for goat IL-6 with a minimum detectable concentration of 1.0 pg/mL. Total serum cortisol concentrations were measured using a solid-phase RIA kit (Diagnostic Products Corp., Los Angeles, CA).

Statistical evaluation was performed using SAS software (SAS Institute, 2004). Analysis of variance for repeated measures was performed using PROC MIXED of SAS. The statistical model included the fixed effect of treatment (3 levels), sampling time (8 levels), the interaction between treatment and sampling time, and the random effect of goat within treatment. In addition, the model applied a first-order autoregressive covariance structure to account for the correlation between measures within a goat. When there was an overall effect of treatment ($P < 0.05$), differences between the least squares means were compared using a *t*-test. Significance was declared at $P < 0.05$.

The procedure of infusion and collection of samples was completed without any complications. The goats in the control group had a relatively stable body temperature throughout the experiment. Intramammary inoculation with *Staph. aureus* produced a marked monophasic febrile response in goats. Rectal temperature started to increase from 2 h and peaked at 12 h (40.9°C; SAB) and 24 h (39.9°C; SAA) postinoculation. All inoculated udders showed a gradual decrease in milk production, changes in milk appearance (particularly appearance of milk clots), and local warmth 24 h after inoculation. *Staphylococcus aureus* was detected in milk collected from all infected udders throughout the study.

Plasma Fe concentrations were lower in group SAA than in the control group at 12 ($P = 0.036$) and 24 h ($P = 0.002$) postinoculation, respectively (Table 1). Plasma Fe concentrations were lower in group SAB than in the control group at 6 ($P = 0.006$), 12 ($P = 0.004$), and 24 h ($P = 0.004$) postinoculation, respectively. Plasma Fe concentrations in groups SAA and SAB had returned to control concentrations 48 h after inoculation. Increased plasma Cu concentrations in group SAB were observed first at 2 h, peaked at 12 h postinoculation (2.73 vs. 1.79 mg/kg in the control group), and

Table 1. Plasma Fe, Cu, Zn, and Mg concentrations at different times in dairy goats following intramammary inoculation with PBS (control) and *Staphylococcus aureus*

Item	Treatments ¹			SEM
	Control	SAA	SAB	
Fe, mg/kg				
0 h	1.32	1.37	1.41	0.164
2 h	1.41	1.67	1.42	0.558
4 h	1.75	1.66	1.54	0.256
8 h	1.56 ^a	1.54 ^a	1.14 ^b	0.138
12 h	1.50 ^a	1.08 ^b	0.73 ^b	0.210
24 h	1.46 ^a	0.98 ^b	0.68 ^b	0.091
48 h	1.40	1.48	1.19	0.420
72 h	1.56	1.57	1.46	0.313
Cu, mg/kg				
0 h	1.85	1.77	1.96	0.261
2 h	1.81 ^b	1.63 ^b	2.50 ^a	0.176
4 h	1.78 ^b	1.61 ^b	2.57 ^a	0.182
8 h	1.82 ^b	1.52 ^b	2.54 ^a	0.150
12 h	1.77 ^b	1.79 ^b	2.73 ^a	0.254
24 h	1.76 ^b	1.69 ^b	2.32 ^a	0.233
48 h	1.91 ^b	1.64 ^b	2.71 ^a	0.128
72 h	1.85 ^b	1.74 ^b	2.77 ^a	0.183
Zn, mg/kg				
0 h	0.72	0.67	0.73	0.160
2 h	0.68 ^b	1.03 ^a	0.83 ^{ab}	0.117
4 h	0.72 ^b	0.71 ^b	0.90 ^a	0.068
8 h	0.67 ^b	1.01 ^a	0.75 ^b	0.086
12 h	0.67	0.63	0.66	0.085
24 h	0.64	0.58	0.72	0.076
48 h	0.73 ^b	0.77 ^b	1.05 ^a	0.117
72 h	0.75 ^b	0.75 ^b	1.04 ^a	0.078
Mg, mg/kg				
0 h	45.38	45.20	43.50	4.133
2 h	37.18 ^b	50.36 ^a	53.58 ^a	5.833
4 h	36.98 ^b	51.94 ^a	42.34 ^b	5.918
8 h	28.92 ^b	53.32 ^a	41.60 ^{ab}	6.825
12 h	24.48 ^c	52.92 ^a	33.28 ^b	3.690
24 h	25.34 ^b	58.06 ^a	38.49 ^b	5.153
48 h	27.38 ^c	53.10 ^a	37.78 ^b	4.275
72 h	25.38 ^c	43.79 ^a	36.14 ^b	3.674

^{a-c}Means within a row with different superscripts differ ($P < 0.05$).¹Treatments (n = 5 goats per group): Control = goats inoculated with PBS; SAA = goats inoculated with 10^4 cfu of *Staph. aureus*; SAB = goats inoculated with 10^8 cfu of *Staph. aureus*.

remained elevated throughout the experiment. In the control group and SAA, plasma Cu concentrations remained relatively stable throughout the experiment and no differences were detected between these 2 groups (Table 1). Increased plasma Zn concentrations were seen in group SAB at 4 ($P = 0.020$), 48 ($P = 0.020$) and 72 h ($P = 0.001$) postinoculation, respectively, compared with the control group (Table 1). Plasma Zn concentrations were greater in group SAA than in the control group at 2 h ($P = 0.013$) and 8 h ($P = 0.002$) postinoculation, respectively. Plasma Mg concentrations in group SAA were generally greater ($P < 0.05$) compared with the control group and group SAB throughout the experiment (except at 2 h; Table 1). Plasma Mg concentrations were greater ($P < 0.05$) in group SAB than in

Table 2. Plasma tumor necrosis factor-alpha (TNF- α) and IL-6 concentrations and serum cortisol concentrations at different times in dairy goats following intramammary inoculation with PBS and *Staphylococcus aureus*

Item	Treatments ¹			SEM
	Control	SAA	SAB	
TNF- α , pmol/L				
0 h	13.88	15.68	13.30	1.895
2 h	13.23	14.84	16.96	2.027
4 h	15.65	16.18	13.10	2.383
8 h	15.62	16.10	13.87	2.704
12 h	13.37	16.38	14.14	2.292
24 h	13.36	14.98	15.35	1.911
48 h	15.67	16.46	15.33	1.975
72 h	15.21	14.14	16.99	2.050
IL-6, pg/mL				
0 h	2,218	2,606	3,093	130.1
2 h	2,068	2,097	2,502	143.7
4 h	1,821 ^b	3,630 ^a	2,692 ^{ab}	221.5
8 h	2,320	2,652	2,370	146.8
12 h	2,063 ^b	2,708 ^{ab}	3,387 ^a	220.3
24 h	2,358 ^b	2,962 ^a	2,135 ^b	186.2
48 h	2,259 ^b	4,748 ^a	4,232 ^a	248.5
72 h	2,273 ^b	3,089 ^{ab}	3,794 ^a	202.8
Cortisol, ng/mL				
0 h	25.71	19.88	10.74	6.453
2 h	9.88	10.06	10.31	1.715
4 h	6.03	10.36	15.83	1.758
8 h	11.25 ^b	30.90 ^b	63.63 ^a	11.279
12 h	19.32 ^b	24.47 ^b	126.56 ^a	27.628
24 h	10.87	16.33	25.59	4.948
48 h	12.41	10.87	8.00	4.173
72 h	9.55	9.99	12.82	3.006

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).¹Treatments (n = 5 goats per group): Control = goats inoculated with PBS; SAA = goats inoculated with 10^4 cfu of *Staph. aureus*; SAB = goats inoculated with 10^8 cfu of *Staph. aureus*.

the control group throughout the experiment (except for 8 and 24 h).

Plasma TNF- α concentrations remained unaltered in all groups throughout the study (Table 2). Plasma IL-6 concentrations were greater in group SAA at 4 ($P = 0.039$), 12 ($P = 0.048$), 24 ($P = 0.047$), and 48 h ($P = 0.007$) than in the control group (Table 2). Plasma IL-6 concentrations were greater in group SAB at 12 ($P = 0.045$), 48 ($P = 0.001$), and 72 h ($P = 0.029$) compared with the control group. Peak concentrations of IL-6 occurred at 48 h in groups SAA and SAB. In group SAB, serum cortisol concentrations increased approximately 5-fold at 6 h (63.6 vs. 11.3 ng/mL in the control group; $P = 0.046$) and 6-fold at 12 h (126.6 vs. 19.3 ng/mL in the control group; $P = 0.009$) postinoculation, respectively, and returned to the baseline concentration 24 h postinoculation (Table 2). Serum cortisol concentrations were relatively constant throughout the study in the control group and group SAA.

The acute phase response is a part of the early defense mechanisms involving the induction of an inflammatory mediator cascade, which is characterized by local

vascular effects and systemic multi-organ effects and metabolic changes (Lohuis et al., 1988). The observations here showed the early local and systemic responses, including local warmth and changes in body temperature and milk appearance in goats following intramammary inoculation with *Staph. aureus*. Release of inflammatory mediators such as proinflammatory cytokines IL-6 and TNF- α from the site of infection may explain how local microbes may cause systemic symptoms in the absence of microbial invasion.

A decrease in plasma Fe concentrations to 67% (group SAA) and 47% (group SAB) of control concentrations was found at 24 h postinoculation. An increase in plasma Cu and Zn to 153 and 143%, respectively (group SAB), of control concentrations at 12 h (Cu) and 48 h (Zn) postinoculation occurred. In contrast, a previous study in cows showed a decrease in serum Fe, Cu, and Zn concentrations to 81, 89, and 83% of preinfection concentrations at 24 h postchallenge with *Staph. aureus* (Middleton et al., 2004). The differences in results may be due to species, but it is more likely because the infection model used in our study was a clinical mastitis model. In contrast, as shown in the previous study, intramammary administration of *Staph. aureus* induced subclinical mastitis. The discrepancy between these studies might be attributable to the lower doses of *Staph. aureus* used in the previous study. An increase in plasma Cu concentrations was observed in rats and hamsters following parenteral administration of endotoxin (Etzel et al., 1982). During acute phase reactions, increased plasma Cu concentrations may be partly due to increased serum concentrations of copper-binding protein. An increase in plasma Mg concentrations to 229% (group SAA) and 152% (group SAB) of control concentrations at 24 h postinoculation was found in the present study. Our results show that altered mineral metabolism occurred in goats in response to intramammary *Staph. aureus* infection. Microbial metabolism requires Fe and Zn, although the role of Zn and its interactions with Fe as a nonspecific host defense against bacterial infections remain unclear. Further studies are needed to elucidate a possible role of these trace metals in protection against, or the development of, *Staph. aureus* mastitis.

Our results show that plasma IL-6 concentrations were greater in groups SAA and SAB than in the control group, whereas there was no significant increase in plasma TNF- α concentrations in either SAA or SAB after inoculation with *Staph. aureus*. Similarly, previous studies reported that there was no increase in the concentrations of TNF- α in milk collected from cows challenged with *Staph. aureus* (Riollet et al., 2000; Bannerman et al., 2004). Some cytokines influence mineral metabolism by inducing synthesis of their transport

proteins. Moreover, Zn is supposed to induce the release of some cytokines such as IL-1 and IL-6 from monocytes. We showed that both Zn and IL-6 increased in the SAB group, whereas IL-6 increased in the SAA group without an increase in Zn. It seems that in addition to Zn, other signals (Akira et al., 2006) may also stimulate the induction of IL-6. Increased serum cortisol concentrations were detected 6 and 12 h after intramammary inoculation with *Staph. aureus*, implying that the activity of the hypothalamic-pituitary-adrenal axis could be affected by intramammary *Staph. aureus* infection.

In conclusion, we show here that following intramammary inoculation with *Staph. aureus*, plasma IL-6 concentrations increased, whereas plasma TNF- α concentrations remained unchanged. Serum cortisol concentrations increased 12 h after intramammary administration of a high dose (10^8 cfu) of *Staph. aureus*. Decreased serum Fe concentrations were observed, but increased serum Cu, Zn, and Mg concentrations were seen following intramammary inoculation with *Staph. aureus*. Our data indicate that mineral metabolic alterations occur during the course of *Staph. aureus* mastitis in the goat. Further studies correlating mineral changes in both milk and blood with inflammation-induced factors (e.g., lactoferrin and ceruloplasmin) and inflammatory mediators would help gain a better understanding of the role of these minerals in *Staph. aureus* mastitis.

REFERENCES

- Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell* 124:783–801.
- Bannerman, D. D., M. J. Paape, J. W. Lee, X. Zhao, J. C. Hope, and P. Rainard. 2004. *Escherichia coli* and *Staphylococcus aureus* elicit differential innate immune responses following intramammary infection. *Clin. Diagn. Lab. Immunol.* 11:463–472.
- Ersikine, R. J., and P. C. Bartlett. 1993. Serum concentrations of copper, iron, and zinc during *Escherichia coli*-induced mastitis. *J. Dairy Sci.* 76:408–413.
- Etzel, K. R., M. R. Swerdel, J. N. Swerdel, and R. J. Cousins. 1982. Endotoxin-induced changes in copper and zinc metabolism in the Syrian hamster. *J. Nutr.* 112:2363–2373.
- Failla, M. L. 2003. Trace elements and host defense: Recent advances and continuing challenges. *J. Nutr.* 133:1443S–1447S.
- Lohuis, J. A., J. H. Verheijden, C. Burvenich, and A. S. van Miert. 1988. Pathophysiological effects of endotoxins in ruminants. 2. Metabolic aspects. *Vet. Q.* 10:117–125.
- Middleton, J. R., C. D. Luby, L. Viera, J. W. Tyler, and S. Casteel. 2004. Short communication: Influence of *Staphylococcus aureus* intramammary infection on serum copper, zinc, and iron concentrations. *J. Dairy Sci.* 87:976–979.
- Riollet, C., P. Rainard, and B. Poutrel. 2000. Differential induction of complement fragment C5a and inflammatory cytokines during intramammary infections with *Escherichia coli* and *Staphylococcus aureus*. *Clin. Diagn. Lab. Immunol.* 7:161–167.
- SAS Institute. 2004. User's Guide: Statistics. Version 9.1. SAS Institute Inc., Cary, NC.
- Sears, P. M., and K. K. McCarthy. 2003. Management and treatment of staphylococcal mastitis. *Vet. Clin. North Am. Food Anim. Pract.* 19:171–185.

- Sladek, Z., D. Rysanek, H. Ryznarova, and M. Faldyna. 2005. Neutrophil apoptosis during experimentally induced *Staphylococcus aureus* mastitis. *Vet. Res.* 36:629–643.
- Wang, J., L. Jiao, J. Ma, C. Wu, K. Wang, and M. Wang. 2007. Effects of intravenous infusion of lipopolysaccharide on plasma micromineral, magnesium, and cytokine concentrations and serum cortisol concentrations in lactating goats. *Am. J. Vet. Res.* 68:529–534.
- Wang, J. F., M. Wang, J. L. Ma, L. G. Jiao, X. Y. Zhou, and J. E. Lindberg. 2006. The influence of intramammary lipopolysaccharide infusion on serum Ca, P, vitamin D, cytokines and cortisol concentrations in lactating sows. *J. Vet. Med. A Physiol. Pathol. Clin. Med.* 53:113–118.
- Weiss, G., H. Wachter, and D. Fuchs. 1995. Linkage of cell-mediated immunity to iron metabolism. *Immunol. Today* 16:495–500.
- Zhu, Y., I. Osterlundh, F. Hulten, and U. Magnusson. 2004. Tumor necrosis factor-alpha, interleukin-6, serum amyloid A, haptoglobin, and cortisol concentrations in sows following intramammary inoculation of *Escherichia coli*. *Am. J. Vet. Res.* 65:1434–1439.