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Immune responses in lactating Holstein cows supplemented with Cu, Mn, and Zn as sulfates or methionine hydroxy analogue chelates

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ABSTRACT

The aim of this study was to compare effects of inorganic sulfate versus chelated forms of supplemental Cu, Mn, and Zn on milk production, plasma and milk mineral concentrations, neutrophil activity, and antibody titer response to a model vaccination. Holstein cows (n = 25) were assigned in 2 cohorts based on calving date to a 12-wk randomized complete block design study. The first cohort consisted of 17 cows that had greater days in milk (DIM; mean of 77 DIM at the start of the trial) than the second cohort of 8 cows (32 DIM at the start of the trial). Diets were formulated to supplement 100% of National Research Council requirements of Cu, Mn, and Zn by either inorganic trace minerals (ITM) in sulfate forms or chelated trace minerals (CTM) supplied as metal methionine hydroxy analog chelates, without accounting for trace mineral contribution from other dietary ingredients. Intake and milk production were recorded daily. Milk composition was measured weekly, and milk Cu, Mn, and Zn were determined at wk 0 and 8. Plasma Cu and Zn concentrations and neutrophil activity were measured at wk 0, 4, 8, and 12. Neutrophil activity was measured by in vitro assays of chemotaxis, phagocytosis, and reactive oxygen species production. A rabies vaccination was administered at wk 8, and vaccine titer response at wk 12 was measured by both rapid fluorescent focus inhibition test and ELISA. Analyzed dietary Cu was 21 and 23 mg/kg, Mn was 42 and 46 mg/kg, and Zn was 73 and 94 mg/kg for the ITM and CTM diets, respectively. No effect of treatment was observed on milk production, milk composition, or plasma minerals. Dry matter intake was reduced for CTM compared with ITM cows, but this was largely explained by differences in body weight between treatments. Milk Cu concentration was greater for CTM than ITM cows, but this effect was limited to the earlier DIM cohort of cows and was most pronounced for multiparous compared with primiparous cows. Measures of neutrophil function were unaffected by treatment except for an enhancement in neutrophil phagocytosis with the CTM treatment found for the later DIM cohort of cows only. Rabies antibody titer in CTM cows was 2.8 fold that of ITM cows as measured by ELISA, with a trend for the rapid fluorescent focus inhibition test. Supplementation of Cu, Mn, and Zn as chelated sources may enhance immune response of early lactation dairy cows compared with cows supplemented with inorganic sources.
Key words: trace mineral, neutrophil, vaccine response

INTRODUCTION

The trace minerals Cu, Mn, and Zn are incorporated into a variety of proteins and enzymes that are involved in a wide range of physiological processes. Preventing trace mineral deficiencies has long been recognized as important in the maintenance of production, reproduction, and health (Miller, 1981; NRC, 2001). As dairy cattle productivity continues to increase, interest has grown in improving trace mineral feeding strategies. Specific areas of research include determining the effect of trace mineral chemistry on mineral retention and dairy cattle health and performance (Nocek et al., 2006; Siciliano-Jones et al., 2008).

Traditionally, Cu, Mn, and Zn supplements have been fed as inorganic compounds such as sulfate salts. In these salts, the trace mineral is associated with sulfate in a dry form but dissociates from the sulfate when hydrated in the digestive tract. Dissociated trace minerals in the reticulo-rumen, omasum, and abomasum can interact with components of digesta to form insoluble or indigestible compounds that pass into feces (McDonald et al., 1996; Spears, 2003). Organic forms of Cu, Mn, and Zn including metal AA chelates, metal complexes, metal methionine hydroxy analog chelates, metal proteinates, and metal propionates have been developed to increase intestinal absorption and mineral bioavailability (Predieri et al., 2005; Wright et al., 2008). A given amount of absorbable trace mineral may, therefore, be supplied with a lower amount of an organic trace mineral compared with an inorganic

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trace mineral. Alternatively, feeding similar levels of organic supplements in place of inorganic supplements may provide increased bioavailable trace minerals to support biological processes.

Copper, Mn, and Zn are required to support animal immune function and health. Indeed, severe or marginal deficiencies in trace minerals, especially Zn, can lead to innate and adaptive immune system dysfunction in a wide variety of species (Fernandes et al., 1979; Sordillo et al., 1997; Erickson et al., 2000; Fraker et al., 2000; Ibs and Rink, 2003; Cui et al., 2004). Copper, Mn, and Zn function in the immune system in several ways, including their activity in antioxidant pathways and in maintaining structural integrity of epithelial barriers against infection (Miller et al., 1993; Spears and Weiss, 2008; Richards et al., 2010). A study of 2,080 commercial dairy and beef herds also found that deficient or marginal plasma Zn concentration was associated with increased incidence of metritis in cows, and deficient or marginal Cu or Zn was associated with increased health disorders in calves (Enjalbert et al., 2006). Finally, as summarized by Andrieu (2008), supplementation with Cu or Zn proteinates compared with inorganic forms has resulted in improved mammary health.

The objective of this experiment was to compare performance, plasma and milk minerals, and measures of innate and adaptive immune function in early lactation cows fed Cu, Mn, and Zn supplied by either inorganic or chelated organic sources for 12 wk.

MATERIALS AND METHODS

Animals, Treatments, and Sampling

All animal procedures were approved by the Agricultural Animal Care and Use Committee at the University of Delaware. Twenty-six Holstein dairy cows (12 primiparous and 14 multiparous) were used in a 12-wk study. Cows were milked and milk yields recorded twice daily at 0630 and 1730 h. To minimize DIM at the start of the trial, animals were enrolled in the study in 2 cohorts based on calving date, with the first cohort starting 1 mo before the second cohort. The first cohort consisted of 17 cows (8 primiparous and 9 multiparous) that were slightly later in lactation (77 ± 33 DIM at the start of the trial) than cohort 2, which consisted of 9 cows (4 primiparous and 5 multiparous) that were 32 ± 11 DIM at the start of the trial. Cows were housed in a barn with 47 sand-bedded freestalls. Cows were trained to use Calan feed gates (American Calan, Northwood, NH) for approximately 2 wk before the start of the trial, and all cows were fed the ration containing inorganic trace minerals during this training period. Cows were fed once daily at 0700 h with a targeted feeding rate of 10% daily refusals, and intake and refusals were recorded daily. For 60 d before expected calving date, heifers and cows were housed in a single group and fed a dry cow ration balanced to contain 23, 58, and 81 mg/kg of Cu, Mn, and Zn, respectively. Following parturition and until the start of the trial, cows were moved to a single lactating group pen and fed a ration balanced to contain 17, 53, and 85 mg/kg of Cu, Mn, and Zn, respectively.

Following the training period, cows were blocked by parity, milk yield, and DIM and randomly assigned to 1 of 2 dietary treatments according to a randomized complete block design. Treatments were dietary supplementation of Cu, Mn, and Zn as either inorganic trace minerals (**ITM**) or chelated trace minerals (**CTM**). Supplemental Cu, Mn, and Zn were supplied as sulfate salts for the ITM ration, and as metal methionine hydroxy analog (2-hydroxy-4-methylthiobutanoic acid, **HMTBa**) chelates (Novus International Inc., St. Charles, MO) for the CTM ration. The ration composition is presented in Table 1 and the trace mineral and vitamin mix composition is presented in Table 2. For ITM, the trace minerals and vitamins were provided by a commercial mix (Renaissance Nutrition Inc., Roaring Spring, PA) that contained Cu, Mn, and Zn as sulfate salts. The CTM trace mineral and vitamin mix was balanced to provide the same levels of minerals and vitamins as the ITM mix, except that Cu, Mn, and Zn were included in the CTM ration as HMTBa chelates (Table 2). Trace mineral and vitamin mixes were balanced to provide 100% of NRC (2001) requirements for Cu, Mn, and Zn without taking into account the mineral contribution from other ration ingredients. Remaining minerals and vitamins were balanced to meet or exceed requirements (NRC, 2001). Cobalt was not included in the trace mineral mix. Monthly composite TMR samples were analyzed for Co in a commercial laboratory (Eurofins Scientific Inc., Des Moines, IA) via inductively coupled plasma atomic emission spectroscopy (method 986.15; AOAC, 2000), and all samples were well above NRC (2001) requirements. The ITM and CTM rations (Table 1) contained 0.09 and 0.07% HMTBa, respectively, to account for most of the HMTBa supplied with the CTM trace mineral and vitamin mix. Corn silage was adjusted to account for the difference. The concentrate mixes including the trace mineral and vitamin mixes were prepared at a commercial facility approximately once per month and transported to the dairy.

Body weights and BCS were measured for 2 consecutive days during wk 0, 4, 8, and 12. Body condition score was evaluated using a scale of 1 to 5, with 1 = thin and 5 = fat (Wildman et al., 1982).

Table 1. Ingredient composition (% DM basis) of the experimental diets

Item	Ration[1]	
	ITM	CTM
Corn silage[2]	49.38	49.40
Alfalfa hay[3]	6.41	6.41
Alfalfa silage[4]	6.31	6.31
Corn grain, ground	7.98	7.98
Citrus pulp	5.29	5.29
Canola meal	5.26	5.26
Soybean meal, extruded and expelled	5.20	5.20
Soybean meal	3.67	3.67
Corn distillers grains	2.92	2.92
Soybean hulls	2.28	2.28
Blood meal	1.11	1.11
Calcium carbonate	0.78	0.78
Rumen inert fat[5]	0.69	0.69
Sodium bicarbonate	0.67	0.67
Molasses	0.62	0.62
Sodium chloride	0.42	0.42
Menhaden fish meal	0.38	0.38
Urea	0.11	0.11
Magnesium oxide	0.10	0.10
Methionine source[6]	0.09	0.07
Trace mineral and vitamin mix[7]	0.33	0.33

[1]Treatments were Cu, Mn, and Zn supplied by inorganic (ITM) or chelated (CTM) trace mineral sources.

[2]Corn silage contained, on average, 7.2% CP, 40.2% NDF, 23.3% ADF, 33.9% starch, 3.6% ash, 5 mg of Cu/kg, 14 mg of Mn/kg, and 17 mg of Zn/kg.

[3]Alfalfa hay contained, on average, 17.8% CP, 47.3% NDF, 37.2% ADF, 8.4% ash, 10 mg of Cu/kg, 30 mg of Mn/kg, and 23 mg of Zn/kg.

[4]Alfalfa silage contained, on average, 21.6% CP, 43.2% NDF, 37.2% ADF, 10.3% ash, 8 mg of Cu/kg, 37 mg of Mn/kg, and 29 mg of Zn/kg.

[5]Energy Booster 100 (MSC, Carpentersville, IL).

[6]Source was 84% 2-hydroxy-4-methylthiobutanoic acid (HMTBa; Novus International Inc., St. Charles, MO).

[7]The composition of trace mineral and vitamin mixes is presented in Table 2.

Total mixed ration samples were collected 3 times per week and stored at −20°C until composited by week. Samples of forages and concentrate mixes were taken weekly. Samples were dried for 48 h in a 60°C forced-air oven. The TMR composite samples were sent to Cumberland Valley Analytical Services (Hagerstown, MD) every 2 wk for wet chemistry analysis of CP (method 990.03; AOAC, 2000), NDF using α-amylase and sodium sulfite (Van Soest et al., 1991), ADF (method 973.18; AOAC, 2000), starch (Hall, 2009), and macrominerals (method 985.01; AOAC, 2000). Trace minerals were analyzed in forage and concentrate samples from 8 different weeks spread throughout the trial. Samples were ground through a 0.2-mm sieve, and 10 g was subsampled from each feed for analysis. Trace minerals were measured using AOAC (2000) method 968.08, with the exception of the use of 6 *M* HCl in place of 3 *M* HCl. Digested samples were analyzed in duplicate using inductively coupled plasma optical emission spectrometry (model 2100 Dual View; PerkinElmer Inc., Waltham, MA).

Milk samples were collected weekly at 2 consecutive milkings and sent to Dairy One Milk Laboratory (Ithaca, NY) for infrared analysis of fat, true protein, MUN, and SCC using a MilkoScan System 4000 (Foss North America, Eden Prairie, MN). Additional 50-mL milk samples were collected into trace mineral-free tubes at 2 consecutive milkings during wk 0 (the final week of the acclimation period) and wk 8 and stored at −20°C until mineral analysis. For trace mineral analysis, milk samples were thawed and composited based on a.m. and p.m. milk weights into single samples for each wk (0 or 8). From each composite sample, 2.5 mL was digested with 2.5 mL of concentrated nitric acid, 0.835 mL of hydrogen peroxide, and 4.165 mL of distilled water. Samples were digested using a Mars 5 Microwave Accelerated Reaction System with digestion occurring in XP 1500 vessels wrapped in a Kevlar sleeve (CEM Corp., Matthews, NC). Digestion took place during two 15-min cycles at 200°C. Digested milk samples were analyzed in duplicate for Cu, Mn, and Zn concentrations using inductively coupled plasma optical emission spectrometry (IRIS Intrepid II XSP; Thermo Scientific, Waltham, MA).

Table 2. Ingredient composition (% DM basis) of the trace mineral and vitamin mixes[1]

Item	Mineral mix[2]	
	ITM	CTM
K_2SO_4 and $MgSO_4$ blend[3]	55.5	50.0
$CaCO_3$	34.0	33.4
$CuSO_4 \cdot 5H_2O$	1.20	0
Cu chelate[4]	0	2.00
$MnSO_4 \cdot H_2O$	1.20	0
Mn chelate[4]	0	3.00
$ZnSO_4 \cdot H_2O$	4.28	0
Zn chelate[4]	0	9.50
Vitamin ADE[5]	1.87	1.87
KCl	1.05	0
MgO	0.67	0
Se source[6]	0.22	0.22
$Ca(IO_3)_2$	0.04	0.04

[1]Trace mineral and vitamin mixes were included in the experimental rations at 0.33% of ration DM.

[2]Treatments were Cu, Mn, and Zn supplied by inorganic (ITM) or chelated (CTM) trace mineral sources.

[3]Consisted of 22.5% S, 18.0% K, and 11.5% Mg (Dynamate; The Mosaic Co., Plymouth, MN).

[4]Metal methionine hydroxy analog chelates (Novus International Inc., St. Charles, MO).

[5]Contained 105,840,000 IU of vitamin A/kg, 26,460,000 IU of vitamin D/kg, and 352,800 IU of vitamin E/kg.

[6]Sodium selenite source provided 30,000 mg of Se/kg.

Blood samples (30 mL) were collected via jugular venipuncture into trace mineral-free EDTA-coated Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) at 0800 h the day before treatments began (wk 0) and at the end of wk 4, 8, and 12 for analysis of plasma mineral concentrations and neutrophil function.

Blood in EDTA tubes was centrifuged at 600 × *g* at 4°C for 30 min, and plasma was removed and stored at −20°C until mineral analysis. Remaining blood was used for neutrophil isolation as described below. For mineral analysis, 200 μL of thawed plasma was added to 9.8 mL of 1% nitric acid and centrifuged at 2,200 × *g* at 4°C for 15 min. Duplicate samples of supernatant were analyzed for Cu and Zn concentrations using inductively coupled plasma mass spectrometry (Agilent 7500 Series ICP-MS; Agilent Technologies Inc., Wilmington, DE).

Neutrophil Chemotaxis, Phagocytosis, and Oxidative Burst

After removal of plasma for mineral analysis, neutrophils were isolated from the remaining blood sample following the procedures of Carlson and Kaneko (1973). Blood was centrifuged at 1,000 × *g* at 4°C for 30 min, and the remaining plasma, buffy coat, and two-thirds of the red cell pack were removed and discarded. Erythrocytes were lysed by adding 18 mL of 4°C hypotonic solution (10.6 m*M* Na_2HPO_4 and 2.7 m*M* NaH_2PO_4) and swirling for 90 s. Isotonicity was then restored by the addition of 9 mL of 4°C hypertonic solution (10.6 m*M* Na_2HPO_4, 2.7 m*M* NaH_2PO_4, and 430 m*M* NaCl) and inverting. Tubes were centrifuged at 800 × *g* at 4°C for 5 min. The supernatant was then decanted and cells were resuspended in 10 mL of Hanks' Balanced Salt Solution (**HBSS**; Mediatech Inc., Manassas, VA). The centrifugation, decanting, and resuspension were repeated twice, except that after the third centrifugation, cells were resuspended in only 2 mL of HBSS. Neutrophil concentration was measured using a Z2 Coulter Counter (Beckman Coulter Inc., Fullerton, CA). Cells were then reconstituted to a concentration of 2×10^6 cells/mL in HBSS containing 5% fetal bovine serum and vortexed. Neutrophil function was assessed using in vitro measures of chemotaxis, phagocytosis, and oxidative burst. All assays were conducted in media comprising HBSS with 5% fetal bovine serum. On average, isolated cells were 96% neutrophils as measured by differential staining and were 95% viable, as indicated by trypan blue exclusion.

For measurement of neutrophil chemotaxis, 28 μL of unsupplemented media or media supplemented with human IL-8 (100 ng/mL, no. I1645; Sigma-Aldrich Co., St. Louis, MO) or recombinant human complement 5a (**C5a**; 10^{-8} *M*, no. C5788; Sigma-Aldrich Co.) was pipetted into the bottom of 48-well chemotaxis chambers (Neuro Probe Inc., Gaithersburg, MD). Fifty-five microliters of neutrophils (1.1×10^5 cells) was pipetted into the top of the chamber, which was separated from the bottom by a 5-μm pore-size filter (Neuro Probe Inc., Gaithersburg, MD). All cow and chemoattractant combinations were measured in duplicate wells. Chambers were incubated at 37°C with 5% CO_2 and 95% relative humidity for 1 h. Following incubation, the concentration of neutrophils in the bottom wells was measured by a Coulter Counter. Migration toward IL-8 or C5a was quantified relative to migration toward no chemoattractant as

$$\text{Chemotaxis} = (\text{neutrophils/mL in IL-8 or C5a wells})/(\text{neutrophils/mL in media alone}) \times 100\%.$$

Carboxylated fluorescent latex beads (Fluoresbrite Yellow Green Carboxylate Microspheres, 1.75 μm, no. 17687; Polysciences Inc., Warrington, PA) were used to measure phagocytosis by bovine neutrophils. Beads were incubated in the dark for 45 min in heat-inactivated pooled cow serum. Five hundred microliters of neutrophils (1×10^6 cells) was added to 5-mL tubes in triplicate. Fifty-five microliters of the incubated serum and beads was added to all tubes, providing 1×10^7 beads and resulting in a 1:10 ratio of neutrophils:beads. Tubes were covered with paraffin film and aluminum foil and incubated at 37°C for 2 h in an incubator shaker. Following incubation, the tubes were centrifuged for 5 min at 1,000 × *g* at 4°C. The liquid was decanted and cells were washed twice with 1 mL of HBSS. After the final wash, the liquid was decanted and the cells were resuspended in 2 mL of 4% paraformaldehyde in PBS. Control tubes containing cells alone, beads alone, and beads added after cells were fixed in paraformaldehyde were also prepared. All tubes were covered in paraffin film and aluminum foil and stored at 4°C until analyzed within 1 wk using a flow cytometer (FACSCalibur; Becton Dickinson). Flow cytometry was conducted using an excitation wavelength of 488 nm and an emission wavelength of 530 nm. The population was gated for neutrophils based on forward and side scatter, and 4,000 events within the neutrophil gate were counted for each sample. A fluorescence histogram was used to differentiate neutrophils that had engulfed ≥1 bead from neutrophils that did not engulf beads. The control tubes were used to set the neutrophil gate, set the recording regions of the fluorescence histogram, and to correct samples for nonspecific fluorescence. Flow cytometry did not differentiate between cells that were viable and nonviable at the time of paraformaldehyde

addition. Results were expressed as percentage of total neutrophils containing ≥1 bead.

For measurement of oxidative burst, 100 μL of neutrophils (2×10^5 cells) was pipetted into quadruplicate wells of a 96-well luminometer plate. One of the quadruplicate wells was used as a nonactivated control and 80 μL of media was added. To the other 3 wells, 80 μL of phorbol myristate acetate was added at a final concentration of 40 n*M* to activate cells. Twenty microliters of luminol was also added to all wells at a final concentration of 0.5 m*M*. The plate was incubated at 37°C and luminescence recorded every 5 min for 2 h using a Gen51.05 Synergy 2 microplate reader (BioTek Instruments Inc., Winooski, VT). Reactive oxygen species production was calculated in arbitrary units as the sum of luminescence measurements over the 2 h minus luminescence of corresponding control wells.

Rabies Vaccination Response

During wk 8 and 12 blood samplings, an additional 30 mL of blood was collected in serum tubes to measure rabies antibody titer. Immediately following the wk 8 blood sample, cows were i.m. injected with 2 mL of rabies vaccine (Pfizer Defensor 3; Pfizer Inc., New York, NY). Serum tubes were kept at room temperature for 2 h to allow clot formation, centrifuged at 1,000 × *g* at 25°C for 30 min, and serum was stored at −20°C until analysis of rabies antibody titer using 2 methodologies. One set of samples was analyzed by rapid fluorescent focus inhibition test (**RFFIT**) in a commercial laboratory (The Rabies Laboratory, Kansas State University, Manhattan, KS). The other serum samples were analyzed for rabies antibody titer by ELISA using the Platelia Rabies II kit (no. 355–1180; Bio-Rad Laboratories Inc., Hercules, CA), following the instructions supplied by the manufacturer for the quantitative determination of anti-rabies antibody titer in serum or plasma. The kit is supplied with a protein A-peroxidase conjugate for detection of anti-rabies-antibody-antigen complexes. However, because protein A only weakly binds to bovine IgG, a protein G-peroxidase was used instead (no. 539322; Calbiochem, La Jolla, CA). All conditions of validation described by kit instructions were met using the protein G conjugate.

Statistics

During wk 8, a multiparous cow assigned to the ITM treatment in the earlier DIM cohort was found unable to stand, did not respond to veterinary intervention, and was euthanized. Her data were excluded from statistical analysis. For the remaining 25 cows, weekly averages were calculated for intake, milk production, and milk composition before analysis. All results were evaluated using the MIXED procedure of SAS (SAS Institute, 2006). Measures of neutrophil chemotaxis, neutrophil oxidative burst, and rabies antibody titer response were $\log_{10}$ transformed before analysis to obtain homogeneity of residual variance. All results except for milk minerals and rabies vaccination response were evaluated using a model including fixed effects of treatment, week, cohort, and parity (primiparous vs. multiparous) and interactions of treatment × week, treatment × cohort, treatment × parity, week × cohort, week × parity, treatment × week × cohort, and treatment × week × parity. Cow within cohort was included as a random effect, week was included as a repeated measure using an autoregressive covariance structure, and data collected the wk before the start of the trial were included as covariates. Milk mineral concentrations at wk 8 were analyzed using a MIXED model including covariate values and fixed effects of treatment, cohort, parity, and all 2- and 3-way interactions. Rabies vaccine response was analyzed using the same model except that no covariate was included in the model and the $\log_{10}$-transformed difference in antibody titer (wk 12 – wk 8) was the dependent variable. Significance was declared at $P \leq 0.05$ and tendencies declared at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Cows on both treatments were fed rations balanced to supplement 275 mg of Cu/d, 350 mg of Mn/d, and 1,400 mg of Zn/d. This level was chosen to supply 100% of NRC (2001) predicted requirements for minerals supplied in sulfate forms without accounting for mineral content of feedstuffs. When all dietary ingredients were included, the ITM ration was formulated to provide 166, 334, and 176% of required absorbable Cu, Mn, and Zn, respectively (NRC, 2001). Similar calculations were not made for the CTM ration because absorption coefficients for the supplements used in the CTM ration have not been established experimentally in dairy cows. Nonetheless, similar to the ITM treatment, the CTM treatment is expected to exceed NRC (2001) requirements for absorbable amounts of these trace minerals. Both TMR were predicted to contain 17, 36, and 76 mg/kg of Cu, Mn, and Zn, respectively, with 10, 13, and 51 mg/kg of Cu, Mn, and Zn provided by supplemental minerals. Analyzed trace mineral content was close to or slightly above predicted values (Table 3). Relative to formulated trace mineral content, the ITM TMR supplied 126% Cu, 118% Mn, and 96% Zn, and the CTM TMR provided 136% Cu, 128% Mn, and 123% Zn. The zinc content of the CTM ration was 29% greater than the Zn content of the ITM ration.

Table 3. Chemical composition of TMR

Item	Ration[1]	
	ITM	CTM
Nutrient, % of DM		
CP	15.7	16.1
NDF	33.2	33.0
ADF	22.1	21.9
Ash	6.8	7.3
Starch	24.3	24.5
NFC	41.6	40.8
TDN	72.5	72.3
Ca	0.80	0.90
P	0.37	0.38
Mg	0.28	0.33
K	1.55	1.54
Na	0.44	0.50
NE_L, Mcal/kg	1.67	1.67
Trace mineral, mg/kg		
Cu	21	23
Mn	42	46
Zn	73	94

[1]Treatments were Cu, Mn, and Zn supplied by inorganic (ITM) or chelated (CTM) trace mineral sources for 12 wk.

The interaction of treatment × week × parity was not significant for any of the measured variables. Interactions of treatment × cohort, treatment × parity, and treatment × week × cohort were not found unless detailed below. Any significant effects of cohort have been interpreted as due to differences in DIM; however, random variables, including differences in environmental conditions or inherent differences in cows assigned to the cohorts, may have contributed to cohort effects. No effects of treatment were observed on milk yield, milk yield/DMI, BW, DMI/BW, BCS, fat percentage, fat yield, protein yield, MUN, or SCS (Table 4). The DMI was reduced in the CTM treatment ($P = 0.03$), but the numeric increase in production efficiency as measured by milk yield/DMI was not significant ($P = 0.17$). Although no effect of treatment was found on milk protein percentage, an interaction of treatment × week ($P = 0.04$) was observed. This interaction was caused by an effect of treatment during wk 10 (3.04% for ITM vs. 2.85% for CTM, $P = 0.01$); treatments were not different during any other week.

Previous studies have found variable results with regard to production response to organic trace mineral sources. A meta-analysis of 22 studies evaluating response to supplementation with Cu, Mn, Zn, and Co as metal AA complexes found that, across studies, the complexed minerals increased milk, milk fat, and milk protein yields by 0.93, 0.04, and 0.03 kg/d, respectively, compared with control treatments (Rabiee et al., 2010). However, individual trials using similar animal numbers to this study only sometimes found production differences among treatments (Rabiee et al., 2010). Production performance responses of beef and dairy cattle to organic compared with inorganic forms of Zn alone have also been variable (Malcolm-Callis et al., 2000; Spears and Kegley, 2002; Nunnery et al., 2007; Cope et al., 2009). Similar to the results of this study using chelated trace minerals, the meta-analysis of Rabiee et al. (2010) also found no effect of complexed trace minerals on SCC. However, as reviewed by Andrieu (2008), several studies found reduced SCC in cows given trace minerals supplemented as proteinates compared with inorganic trace minerals.

The decrease in DMI with CTM in this trial differs from results from other trials that typically found no effect of trace mineral source on DMI (Spears and Kegley, 2002; Wright and Spears, 2004; Nunnery et al., 2007; Vázquez-Añón et al., 2007; Cope et al., 2009; Hackbart et al., 2010). One of the reasons for this effect on DMI is due to BW, which was numerically greater for cows

Table 4. Effect of dietary mineral source on intake, milk production, milk composition, and BW

Item	Treatment[1]		SEM	*P*-value	
	ITM	CTM		Treatment	Treatment × week
Milk yield, kg/d	41.9	41.4	0.9	0.70	0.92
DMI, kg/d	25.5	24.1	0.4	0.03	0.81
Milk yield/DMI	1.64	1.70	0.03	0.17	0.27
BW, kg	643	636	6	0.41	0.70
DMI/BW, g/kg	40.2	39.0	0.7	0.20	0.53
BCS	2.65	2.67	0.10	0.87	0.36
Fat, %	3.64	3.62	0.08	0.88	0.37
Fat, kg/d	1.51	1.49	0.04	0.63	0.82
Protein, %	2.97	2.92	0.04	0.41	0.04
Protein, kg/d	1.22	1.20	0.03	0.58	0.99
MUN, mg/dL	10.9	10.8	0.4	0.78	0.52
SCS	2.59	2.70	0.20	0.72	0.14

[1]Treatments were Cu, Mn, and Zn supplied by inorganic (ITM) or chelated (CTM) trace mineral sources for 12 wk.

on the ITM treatment; when DMI was expressed relative to BW, the intake difference between treatments was no longer significant ($P = 0.20$). In addition, the reduced DMI for cows on the CTM treatment did not result in differences in performance. We attribute the treatment effect we observed to inherent differences among cows randomly assigned to the 2 treatments and do not expect this effect to be repeatable.

Plasma Mn was not measured due to its low concentration in plasma, but plasma Cu and Zn concentrations were not affected by treatment (Table 5). Studies evaluating effects of source and level of dietary Zn on plasma Zn in dairy and beef cattle similarly found no effect (Malcolm-Callis et al., 2000; Spears and Kegley, 2002; Nunnery et al., 2007; Cope et al., 2009). When calves were supplemented with Zn at levels well above requirements, plasma Zn concentrations increased in response to dietary Zn level and in response to substitution of proteinate or AA chelated sources for inorganic sources (Kincaid et al., 1997; Wright and Spears, 2004). Plasma concentrations of Cu and Zn in cattle are considered sufficient at >9 μM (572 μg/L) and >600 μg/L (Suttle, 2010). Plasma Cu and Zn concentrations for cows on this study were well above these thresholds, indicating sufficient Cu and Zn status in animals on both diets, which likely contributed to the lack of response.

Milk Mn and Zn were unaffected by treatment (Table 5). Milk Cu concentration was increased by the CTM treatment ($P = 0.004$) but was also affected by the interactions of treatment × cohort ($P = 0.008$) and treatment × parity ($P = 0.05$). In the later DIM cohort, no difference between treatments (82 μg/L for ITM and 83 μg/L for CTM, $P = 0.77$) was observed, but CTM increased milk Cu in the earlier DIM cohort (32 vs. 68 μg/L, $P = 0.001$). The treatment × parity interaction was caused by no effect of treatment in primiparous cows (66 μg/L for ITM vs. 73 μg/L for CTM, $P = 0.38$) but increased milk Cu in response to CTM in multiparous cows (48 vs. 78 μg/L, $P < 0.001$). Milk mineral concentrations were within the range of previous reports (Eppard et al., 1985; Anderson, 1992). The treatment × cohort and treatment × parity interactions suggest that early lactation cows and multiparous cows were most responsive to increased dietary Cu availability. Previous research has shown milk Cu concentrations have significantly or numerically decreased when Cu was not supplemented or following a Cu-depleting period (Engel et al., 1964; Sol Morales et al., 2000).

Innate immune response was measured using several in vitro neutrophil function assays that were conducted following 0, 4, 8, and 12 wk of treatment (Table 6). The chemotaxis assay evaluated potential for neutrophils to migrate toward different chemokines that can be produced by leukocytes and tissues during infection. The phagocytosis assay evaluated the potential for neutrophils to engulf pathogens, which in this case were represented by carboxylated fluorescent beads. Finally, we measured generation of reactive oxygen species during oxidative burst, a pathogen-killing function of neutrophils. Although the magnitude of these measures cannot be quantitatively related to neutrophil activity in vivo, studies have shown that cows with poorer-functioning neutrophils as assessed in vitro had increased mastitis severity and were more likely to develop retained placenta (Kremer et al., 1993; Kimura et al., 2002). The lack of treatment effects on any of the neutrophil measures indicates that overall neutrophil function was not affected by trace mineral form. However, an interaction of treatment × cohort × week for phagocytosis ($P = 0.02$) suggests a benefit of the

Table 5. Effect of dietary mineral source on plasma and milk mineral concentrations

	Treatment[1]			*P*-value	
Item	ITM	CTM	SEM	Treatment	Treatment × week[2]
Plasma					
Cu, μg/L	1,262	1,362	47	0.16	0.67
Zn, μg/L	1,024	971	48	0.46	0.54
Milk					
Cu, μg/L	56	76	4	0.004[3]	—
Mn, μg/L	19	28	9	0.52	—
Zn, μg/L	3,560	3,645	118	0.62	—

[1]Treatments were Cu, Mn, and Zn supplied by inorganic (ITM) or chelated (CTM) trace mineral sources for 12 wk.

[2]Plasma mineral concentrations were measured following 0, 4, 8, and 12 wk on treatment, and milk minerals were measured following 0 and 8 wk on treatment. Statistical models included 0-wk measures as covariates.

[3]Also significant were interactions of treatment × cohort ($P = 0.008$) and treatment × parity ($P = 0.05$). In the later DIM cohort, no difference was observed between treatments (82 μg/L for ITM and 83 μg/L for CTM, $P = 0.77$), but CTM increased milk Cu in the earlier DIM cohort (32 vs. 68 μg/L, $P = 0.001$). No effect of treatment was observed in primiparous cows (66 μg/L for ITM vs. 73 μg/L for CTM, $P = 0.38$), but CTM increased milk Cu in multiparous cows (48 vs. 78 μg/L, $P < 0.001$).

Table 6. Effect of dietary mineral source on in vitro measures of neutrophil function and rabies vaccination response

	Treatment[1]			*P*-value	
Item	ITM	CTM	SEM	Treatment	Treatment × week
Neutrophil measure[2]					
Chemotaxis to IL-8[3]	2.36	2.39	0.04	0.57	0.43
Chemotaxis to C5a[3]	2.39	2.37	0.05	0.81	0.90
Phagocytosis[4]	18.6	21.6	1.4	0.15[5]	0.18
Oxidative burst[6]	4.37	4.34	0.03	0.55	0.39
Rabies antibody titer[7]					
ELISA, $\log_{10}$	0.61	1.07	0.11	0.007	—
RFFIT, $\log_{10}$	1.22	1.65	0.17	0.09	—

[1]Treatments were Cu, Mn, and Zn supplied by inorganic (ITM) or chelated (CTM) trace mineral sources for 12 wk.

[2]Neutrophil measures were evaluated following 0, 4, 8, and 12 wk on treatment, with wk 0 included as a covariate.

[3]Neutrophil migration through a 5-μm pore membrane toward unsupplemented media alone or media supplemented with human IL-8 (100 ng/mL) or recombinant human complement 5a (C5a; 50 ng/mL) was measured following 1-h incubation. Results presented as $\log_{10}$(neutrophils per milliliter in IL-8 or C5a/neutrophils per milliliter in media alone × 100%).

[4]Phagocytosis is presented as the percent of neutrophils that engulfed 1 or more fluorescent carboxylated latex beads following 2-h incubations.

[5]An interaction of treatment × cohort × week was observed for phagocytosis ($P = 0.02$). For the later DIM cohort, phagocytosis was similar for both treatments during covariate wk 0 (15.5%), greater for CTM than ITM cows during wk 4 (25.4 vs. 15.6%, $P = 0.002$) and 12 (28.9 vs. 21.7%, $P = 0.02$), with no difference at wk 8 (27.6% vs. 23.5%, $P = 0.17$). For the earlier DIM cohort, no differences were observed between treatments at any week.

[6]Neutrophils were activated with 40 n*M* phorbol myristate acetate and incubated with 0.5 m*M* luminol. Oxidative burst was measured by recording luminescence (arbitrary units) every 5 min for 2 h. Results are presented as $\log_{10}$(sum of luminescence measures over 2 h).

[7]Rabies vaccination (Pfizer Defensor 3; Pfizer Inc., New York, NY) was administered at wk 8 and serum samples were collected at wk 8 and 12. Rabies antibody titer in serum samples was measured in arbitrary units by ELISA and by rapid fluorescent focus inhibition test (RFFIT), and the log-transformed difference between wk 12 and 8 values was used for statistical analysis.

CTM treatment in the cohort of later-DIM cows. For those cows, both treatments started at the same level during covariate wk 0 (15.5%), but phagocytosis by neutrophils of CTM cows was greater than ITM cows during wk 4 (25.4 vs. 15.6%, $P = 0.002$) and 12 (28.9 vs. 21.7%, $P = 0.02$), with no difference at wk 8 (27.6% vs. 23.5%, $P = 0.17$). For the earlier DIM cohort, no differences occurred between treatments at any week.

We had suspected that the CTM treatment would enhance neutrophil function. Compared with animals that were not supplemented, supplementation with Cu or Zn has been shown to enhance neutrophil function or improve response to a disease challenge in dairy and beef cattle (Chirase et al., 1991; Xin et al., 1991; Scaletti et al., 2003). However, Xin et al. (1991) found no effect of dietary Cu on neutrophil phagocytosis of *Staphylococcus aureus* despite an improvement in neutrophil kill of *Staph. aureus*, and Arthington et al. (1995) found no effect of Cu level or source on *Staph. aureus* kill by neutrophils from beef heifers. In the current experiment, cows on both treatments were fed at or above NRC (2001) predicted requirements for Cu, Mn, and Zn, whereas many of the above cited studies compared response relative to a deficient diet. Diminishing responses with increasing supplementation level may have led to the lack of treatment differences observed in this study. One study using the same HMTBa chelated mineral sources as used in this trial evaluated the effect of feeding beef cattle a diet balanced to meet trace mineral requirements using inorganic mineral sources or the same diet with increased Cu, Mn, and Zn provided by the chelated source (Vázquez-Añón et al., 2007). In that experiment, water had high sulfate concentrations, which likely caused mineral antagonisms, and supplementation with the chelated trace mineral sources reduced morbidity and mortality (Vázquez-Añón et al., 2007).

Following 8 wk on treatment, cows were administered a killed rabies vaccination. This model vaccination was chosen to allow for exposure to a novel antigen and elicit a primary vaccine response. Rabies antibody titers were assessed in serum samples collected following 12 wk on treatment because maximum antibody response typically occurs 4 wk following vaccination (National Association of State Public Health Veterinarians Committee, 2008). Prevaccination (wk 8) rabies antibody

titers were low for all cows except for 1 cow whose data were excluded from the statistical analysis. Cows fed CTM had increased antibody titer response to vaccination compared with cows fed ITM, as indicated by a significant difference as assessed by the ELISA ($P = 0.007$) and a trend with the RFFIT ($P = 0.09$; Table 6). The reverse-transformed least squares means from Table 6 indicate the magnitude of the difference in vaccination response. Response of CTM cows was 2.9 and 2.7 times that of ITM cows as measured by ELISA (4.1 for ITM vs. 11.8 for CTM, arbitrary units) and RFFIT (16.4 for ITM vs. 44.5 for CTM, arbitrary units), respectively.

It has long been established that trace mineral (particularly Zn) status affects adaptive immune function in monogastric species. For example, Zn deficiency has been shown to affect T cell and antibody responses in rodents and humans (Beach et al., 1979; Fernandes et al., 1979; Fraker et al., 2000). A study with a similar design to ours fed gilts inorganic or HMTBa chelated sources of Cu, Zn, and Mn, and the measured response to a model vaccination was enhanced in animals fed the chelated sources at both 4 and 8 wk after vaccination (Richards et al., 2010). Effects of trace minerals on measures of adaptive immune function have been less consistent in cattle. In vitro measures of lymphocyte blastogenic response in cattle are generally not enhanced by supplementation with Cu or Zn (Arthington et al., 1995; Kincaid et al., 1997; Spears and Kegley, 2002). Additionally, Cu supplementation in deficient calves inconsistently affected various measures of immune function (Ward et al., 1997). Spears et al. (1991) found that stressed beef steers supplemented with an AA chelated Zn source tended to have greater antibody titer response to a bovine herpesvirus-1 vaccination than unsupplemented steers, although no difference in response to parainfluenza$_3$ vaccination was observed. However, supplementation of Zn as an oxide or proteinate did not affect antibody response to infectious bovine rhinotracheitis vaccine or skinfold thickness response to intradermal phytohemagglutinin compared with unsupplemented steers (Spears and Kegley, 2002). Similarly, Nunnery et al. (2007) found no effect of dietary Zn level or source on antibody titer response to an ovalbumin vaccination regimen in beef heifers. The general lack of favorable responses in cattle might be explained by difficulties to induce deficiencies, perhaps due to underprediction of length of washout periods needed, overprediction of requirements, or underprediction of absorption factors. Given the reported inconsistencies in immune function in cattle in response to trace mineral supplementation, the benefit of the CTM treatment on vaccination response in the current study is striking, especially due to the fact that both treatments supplied above the NRC requirements for Cu, Mn, and Zn.

CONCLUSIONS

Compared with the ITM treatment, cows fed CTM had increased milk Cu concentration and increased antibody titer response to a model vaccination. Treatment did not affect milk production, plasma minerals, or most measures of neutrophil function. Providing more bioavailable trace mineral sources may enhance adaptive immune function compared with inorganic sources.

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