Short communication: Effects of supplementing diets of Holsteins with copper, zinc, and manganese on blood neutrophil function

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ABSTRACT

The effects of supplementing diets with sulfate or glycinate Cu, Zn, and Mn on blood neutrophil function were examined in 27 late-lactation Holstein cows having a mean ($\pm$ standard deviation) days in milk at time of neutrophil assays of 216 ± 31 d. Cows were assigned to 9 blocks of 3 and were grouped by parity, milk production, and days in milk. Cows within each block were randomly assigned to 1 of 3 treatments: (1) control diet devoid of supplemental Cu, Zn, and Mn; (2) diet supplemented with Cu, Zn, and Mn via sulfates; and (3) diet supplemented with Cu, Zn, and Mn via glycinate form. All cows were initially fed a control total mixed ration with basal mineral concentrations of 8 mg/kg of Cu, 35 mg/kg of Zn, and 35 mg/kg of Mn for 30 d. During the treatment period, cows fed diets with mineral supplementation via sulfates or glycinate forms had target total dry matter dietary concentrations of 18 mg/kg of Cu, 60 mg/kg of Zn, and 60 mg/kg of Mn for 30 d. Control cows were fed the control diet devoid of supplemental minerals for an additional 30 d. In vitro neutrophil functions were measured after 30 d on experimental or control diets. Percentage of neutrophils phagocytizing, intracellular kill, and phagocytic index did not differ among treatments. Serum concentrations of Cu, Zn, and Mn were also not affected by dietary treatment after 30 d. Results from this study demonstrated that dietary Cu, Zn, and Mn supplemented either as sulfates or glycinate form for 30 d had no effect on either in vitro blood neutrophil function or serum concentrations of Cu, Zn, and Mn in late-lactation Holstein cows.

Key words: copper, zinc, manganese, neutrophils

Short Communication

Many factors contribute to the functional status of bovine neutrophils that ultimately affect mammary

gland host defenses and prevention of mastitis. Nutritional status of the cow influences host defenses and effects the duration and severity of disease (Scaletti et al., 2003; Goff, 2006; Heinrichs et al., 2009). Microminerals, such as Cu, Zn, and Mn, have been shown to be beneficial in improving udder health by reducing SCC and decreasing the incidence of mastitis in the periparturient and early lactation periods (Scaletti et al., 2003; Siciliano-Jones et al., 2008). Copper, Zn, and Mn are essential for the functionality of many enzymes and structural and cellular proteins (Nocek et al., 2006). The essential nature of these minerals in the antioxidant role of enzymes mediating phagocytosis and killing of bacteria by bovine neutrophils was recently reviewed by Overton and Yasui (2014). Traditionally, microminerals have been supplemented via sulfates; however, organic supplementation has been reported to increase bioavailability and absorption of microminerals in the gut (Andrieu, 2008). Enhancing mineral absorption may have a positive effect on neutrophil function due to increased accessibility to minerals in blood. Studies have shown that organic supplementation of microminerals has been associated with improvements in milk yield, clinical udder score, and *Escherichia coli* counts in milk compared with inorganic and unsupplemented groups following an *E. coli* challenge in early-lactation animals (Scaletti and Harmon, 2012). Although the influence of dietary supplementation of various essential minerals on host defenses and mammary health in early-lactation cows have been reported, little information is available on the benefits of supplementing dietary inorganic and organic sources of minerals on health parameters, including neutrophil function, in late-lactation cows. Therefore, the objective of the current study was to examine the effects of sulfate or glycinate dietary supplementation of Cu, Zn, and Mn in late-lactation cows on in vitro bovine neutrophil phagocytosis and intracellular kill.

Twenty-seven late-lactation Holstein cows in the Ohio Agriculture Research and Development Center Krauss Dairy Herd were used to measure the effects of Cu, Zn, and Mn supplementation on bovine neutrophil function. All procedures using animals were approved through The Ohio State Institutional Animal

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Use and Care Committee (Columbus, OH), Animal Use Protocol: 2014A00000059. Cows were assigned to 9 blocks of 3 based on parity, milk production, and DIM. Four blocks were composed of primiparous cows, and 5 blocks were composed of multiparous cows. Average ($\pm$ SD) milk production was 31.4 ± 6.3 kg during the pretreatment period and DIM was 216 ± 31 d at time of neutrophil assays. Blocks of cows were moved to the pretreatment pen on alternating days over an 18-d period so that each block was sampled on a separate day for neutrophil assays corresponding to d 31 on dietary treatments. Cows were fed a TMR once daily through the pretreatment and treatment periods with a target of 3 to 5% refusal. During the pretreatment period, all cows were fed a diet without supplementation with a commercial source of Cu, Zn, and Mn. Dietary concentrations of Cu, Zn, and Mn were 8.0, 35.0, and 35.0 mg/kg of dietary DM, respectively, for 30 d and cows were housed in freestalls. After this period, cows were moved into tiestalls and placed on 1 of the 3 treatment diets for an additional 30 d (Table 1). Cows within blocks were randomly assigned to 1 of 3 treatments by pulling slips of paper out of a container: (1) devoid of supplemental Cu, Zn, and Mn; (2) supplemented with Cu, Zn, and Mn via sulfates; and (3) supplemented with Cu, Zn, and Mn via glycinate form (B-Traxim, Pancosma, Geneva, Switzerland). The unsupplemented treatment cows, which served as the control, remained on basal control Cu, Zn, and Mn dietary concentrations. The sulfate and glycinate treatments were supplemented with target total dietary concentrations of 18.0, 60.0, and 60.0 mg/kg of DM of Cu, Zn, and Mn, respectively. Details of ingredient composition of diets and nutrient composition of diets and forages supplied by diets are in Table 2. Feed component samples were collected weekly and DM (100°C oven for 24 h) was calculated and recorded. Feed samples were kept frozen at -20°C until the trial was complete. Once the trial was completed, composite samples were prepared for each feed component: corn silage, alfalfa silage, dry hay, and 3 treatment grain mixes. Composite samples were composed of 2 to 4 consecutive sample dates and sent to a commercial analytic laboratory (Cumberland Valley Analytical Services; Hagerstown, MD) for analyses.

Blood (50 mL) was collected from each animal in a block on the same day via jugular vein with a 16-gauge, 1.5-in. needle. Neutrophils were isolated according to Carlson and Kaneko (1973). Blood was collected in sterile 60-mL syringes (BD Luer-Lok, Becton Dickinson Microbiology Systems, Cockeysville, MD) containing 5 mL of EDTA anticoagulant (0.7% NaCl, 1.5% EDTA disodium salt; dehydrate, 0.0132 M KH_2PO_4 and 0.0132 M Na_2HPO_4 ; adjusted to pH 6.6). Forty milliliters of

blood was dispensed into 50-mL conical bottom centrifuge tubes (Corning Inc., Corning, NY) and centrifuged (Sorvall RT7, RTH-750 rotor; Newtown, CT) for 45 min at 4°C and $1,294 \times g$. The plasma, buffy coat, and upper 2 mL of red blood cells were discarded, leaving approximately 8 mL of packed cells remaining. Remaining red blood cells were lysed by adding 30 mL of 0.03 M NaCl to the tube and mixing for 90 s by hand. Eight milliliters of 0.63 M NaCl was added and mixed to bring saline solution back to equilibrium. After lysis, cells were centrifuged for 15 min at 4°C and $748 \times g$. The supernatant was removed, neutrophil pellet was washed with 5 mL of Hanks' Balanced Salt Solution (HBSS; modified, without phenol red and sodium bicarbonate; Sigma-Aldrich, St. Louis, MO) and then centrifuged again for 15 min at 4°C and $748 \times g$. Again, the supernatant was removed and 1 mL of HBSS was added to resuspend the pellet. Viability of isolated cells was measured using a trypan blue exclusion procedure. Briefly, 0.05% trypan blue (Sigma-Aldrich) solution was added to 10 μL of neutrophil suspension, and 100 cells mounted on a hemocytometer (Bright-line hemocytometer; Reichert, Buffalo, NY) were counted at

Table 1. Ingredient composition of the diet¹ (% of DM) for cows fed unsupplemented control diets or sulfate or glycinate Cu-, Zn-, and Mn-supplemented diets for 30 d

Ingredient	Control	Sulfate	Glycinate
Corn silage	29.0	29.0	29.0
Alfalfa silage	29.0	29.0	29.0
Alfalfa hay	5.0	5.0	5.0
Corn dried distillers grains	9.2	9.2	9.2
Ground corn	19.2	19.2	19.1
Soybean meal, 48% CP	6.5	6.5	6.5
Animal/vegetable fat	0.36	0.36	0.36
Limestone	0.65	0.65	0.65
Magnesium oxide	0.05	0.05	0.05
Iodized salt	0.42	0.42	0.42
Selenium premix ²	0.14	0.14	0.14
Vitamin mix ³	0.46	0.18	0.18
Cu sulfate	—	0.003	—
Zn sulfate	—	0.006	—
Mn sulfate	—	0.006	—
Glycinate Cu ⁴	—	—	0.003
Glycinate Zn ⁴	—	—	0.009
Glycinate Mn ⁴	—	—	0.010

¹The control diet was fed to all cows for 30 d before the experiment and to the control group during the 30-d experiment. The sulfate and organic treatments provided all supplemental Cu, Zn, and Mn as either sulfate or as glycinate and were fed only during the experimental phase.

²Sodium selenate, 200 mg/kg.

³Contained 735 kIU of vitamin A/kg, 270 kIU of vitamin D/kg, 4,400 IU of vitamin E, and 135 mg of biotin (Rovimix Biotin, DSM Nutritional Products, Heerlen, the Netherlands) per kilogram.

⁴B-Traxim 2C (Pancosma S. A., Geneva, Switzerland). The products contained 270,000, 260,000, and 220,000 mg of Cu, Zn, and Mn/kg, respectively.

Table 2. Nutrient composition of diet and ingredients for cows fed unsupplemented control diets or sulfate or glycinate Cu-, Zn-, and Mn-supplemented diets for 30 d

Item	Total			Component ¹					
	Control	Sulfate supplemented	Glycinate supplemented	Corn silage	Alfalfa silage	Alfalfa hay	Grain mix sulfate	Grain mix control	Grain mix glycinate
DM, %	59.3	59.0	59.7	36.7	40.6	86.4	87.2	88.0	89.0
OM, %	92.4	92.3	92.2	95.8	88.8	89.7	92.8	92.9	92.5
NDF, %	28.4	28.3	28.2	34.6	39.0	38.0	13.6	14.0	13.4
CP, %	17.0	17.3	17.3	6.7	20.9	19.2	22.4	21.7	22.6
Ca, %	0.97	0.96	0.99	0.20	1.26	1.45	1.25	1.28	1.34
P, %	0.45	0.44	0.44	0.30	0.40	0.36	0.60	0.61	0.60
Mg, %	0.29	0.28	0.29	0.17	0.33	0.31	0.33	0.36	0.35
K, %	1.89	1.88	1.90	1.25	3.43	2.52	1.07	1.10	1.12
S, %	0.28	0.28	0.28	0.11	0.30	0.25	0.40	0.40	0.41
Na, %	0.24	0.22	0.25	0.01	0.06	0.08	0.54	0.58	0.60
Fe, mg/kg	225	222	224	99	504	169	105	112	109
Cu, mg/kg	9	17	20	6	13	9	29	8	37
Zn, mg/kg	41	59	66	33	34	29	104	53	121
Mn, mg/kg	41	54	58	33	69	54	60	23	70

¹All ingredients other than corn silage, alfalfa silage, and alfalfa hay were mixed and analyzed as a grain mix.

40× magnification. Blue and clear cells were counted as dead and live, respectively. Total cell count was determined by counting all cells in 4 fields on a hemocytometer. A differential stain (Protocol HEMA 3 Stain Set; Fisher Scientific Company LLC, Middletown, VA) was completed to determine percentage of neutrophils in the preparation. This was measured by counting first 100 cells observed. The number of viable neutrophils was determined by total number of cells multiplied by viability and percentage of neutrophils [(total cells) × (viability) × (% neutrophil)]. Cell preparations averaged (±SD) 96.9 ± 1.5% viable cells and 94.2 ± 3.4% neutrophils. Concentrations of viable PMNL were adjusted to 4.0 × 10⁶ viable neutrophils/mL of HBSS.

Escherichia coli 487, originally isolated from a mammary gland of a cow with clinical mastitis, was the bacterial strain used for the bacterial phagocytosis assay. An overnight culture of *E. coli* 487 was prepared by inoculating 0.1 mL of stock culture into 12 mL of tryptic soy broth and incubating overnight in a gyratory shaker at 100 rpm and 37°C. The bacteria were then centrifuged (Sorvall RC-5B Refrigerated Super-speed Centrifuge, SS-34 rotor; DuPont Instruments, Wilmington, DE) for 20 min at 4°C and 5,049 × *g*. The pellet was resuspended into 12 mL of HBSS solution and diluted to a 70% transmission at 540 nm (Genesys 6 Spectrophotometer; Thermo Electron Corporation, Madison, WI), corresponding to approximately 8.0 × 10⁶ cfu. Bacteria were serially diluted and spotted (10 µL × 4) on MacConkey (Becton Dickinson Microbiology Systems) agar plates and incubated at 37°C for 24 h to confirm the total count of *E. coli*. Bacteria were opsonized with 10% heat-inactivated bovine serum for 20 min at 20°C and placed on ice. Bovine serum used

for opsonization was collected and pooled from 12 lactating cows.

Neutrophil assays were prepared in a 4:1 [average (±SD) 3.78 ± 1.1:1] bacteria-to-neutrophil ratio (Weiss and Hogan, 2005). Assays were completed in duplicate for each cow within each block. One milliliter of opsonized bacteria was mixed with 0.5 mL of neutrophil suspension in sterile 12 × 75 mm culture tubes with closures. Assays were incubated at 37°C for 1.5 h at 100 rpm on a gyratory shaker. After incubation, samples were diluted 2:1:1 as 50 µL of assay suspension, 25 µL of acridine orange (1.4 mg/10 mL of PBS, Sigma-Aldrich), and 25 µL of crystal violet (5 mg/10 mL of PBS, Fisher Scientific, Fair Lawn, NJ). Wet mount slides were prepared and the number of live (green bacterial cells) and dead (red bacterial cells) bacterial cells were counted for the first 25 neutrophils visible, using the 1,000× oil immersion lens under UV light (Nikon Fluorescence Microscope; Nikon Inc., Garden City, NY). Neutrophils were counted by moving the microscope stage left to right, and observation fields were not repeated. Neutrophil count was completed in duplicate for each cow within each block. Assays were completed within the same day for each individual block to minimize bacterial variability within each block. Assays were blinded so that the individual counting the neutrophils and bacteria had no knowledge of treatments and respective cows. Variables measured were percent neutrophils phagocytizing, neutrophil intracellular kill, and phagocytic index. Percent neutrophils phagocytizing was the number of neutrophils with internalized bacteria divided by the sum of neutrophils with internalized bacteria and neutrophils without internalized bacteria multiplied by 100. Neutrophil intracellular kill

was the number of dead intracellular bacteria divided by the number of dead and live intracellular bacteria multiplied by 100. Last, phagocytic index was the number of phagocytized bacteria divided by the number of neutrophils with internalized bacteria and neutrophils without internalized bacteria.

Six milliliters of blood was taken via tail vein on the first and last days of treatment period from each cow at approximately 5 h after feeding and centrifuged for 10 min at 4°C and $5,049 \times g$. Serum was then removed in 1-mL aliquots and placed in plastic snap vials. Vials were frozen at -20°C and stored until all serum samples were collected. Blood serum was sent to a commercial diagnostic laboratory (Diagnostic Center for Population and Animal Health, Lansing, MI) to be analyzed for trace mineral (Cu, Zn, and Mn) concentrations in the blood. Elemental analysis was completed by methods of Wahlen et al. (2005). Trace mineral concentrations did not differ ($P > 0.05$) among groups the first day of the treatment period with mean ($\pm$ SD) serum concentrations for all cows, at 0.76 ± 0.06 µg/mL, 1.30 ± 0.09 µg/mL, and 1.97 ± 1.40 ng/mL for Cu, Zn, and Mn, respectively.

Neutrophil function data and blood serum concentrations for Cu, Zn, and Mn were analyzed using least squares ANOVA. Models included the main effects of block and diet treatments. A priori comparison of treatments for neutrophil and blood serum data were (1) unsupplemented versus sulfate plus glycinate forms and (2) sulfate versus glycinate forms. Correlations among variables were determined using Pearson correlation coefficient. Statistical analyses were performed using the CORR, GLM procedures in SAS Version 9.4 (SAS Institute Inc., Cary, NC).

Dietary supplementation of Cu, Zn, and Mn via sulfate or glycinate forms for 30 d had no effect on percentage of neutrophils phagocytizing, intracellular kill, or phagocytic index (Table 3). Previous studies have shown varying effects of micronutrient supplementation of diets on bovine host defenses in cows during early or peak lactation. Scaletti et al. (2003) investigated neutrophil function of cows fed diets supplemented with Cu sulfate versus no supplemental Cu. Copper-supplemented animals had lower bacterial counts in milk of challenged glands, as well as lower SCC and lower clinical udder scores than the unsupplemented animals after *E. coli* intramammary challenge. Nemec et al. (2012) investigated differences in neutrophil function between cows fed inorganic and organic sources of Cu, Mn, and Zn. Similar to results of the current trial, Nemec et al. (2012) found no differences between inorganic and organic forms relating to neutrophil chemotaxis, phagocytosis, or oxidative burst. A primary difference in designs of the Scaletti et al. (2003) and Nemec et al. (2012) trials compared with the current study was the stage of lactation for experimental animals. Both of the previous trials used cows in early or peak lactation and the current study used late-lactation cows. Supplementation of micronutrients is often based on alleviation of the depression of host defenses related to decreases in DIM, decreases in micronutrient concentrations in blood, and the physiological changes related to micronutrient deficiencies occurring at parturition (reviewed by Overton and Yasui, 2014). In contrast, dietary supplementation with either sulfate or glycinate forms of Cu, Zn, and Mn did not affect blood concentrations of these minerals or in vitro blood neutrophil responses in late-lactation cows in the current trial.

Table 3. Contrast of in vitro neutrophil functions and blood mineral concentrations for cows fed (1) unsupplemented control diets compared with those fed diets supplemented with Cu, Zn, and Mn in sulfate and glycinate forms (Suppl) and (2) diets supplemented with Cu, Zn, and Mn by either sulfate or glycinate forms

Measurement		Control (n = 9)	Suppl (n = 18)	P-value	Sulfate (n = 9)	Glycinate (n = 9)	P-value
% Phag ¹	Mean	73.4	74.7	0.59	73.2	76.2	0.52
	SE	2.2	1.3		1.2	2.3	
% Killed ²	Mean	55.60	58.24	0.60	60.70	55.78	0.69
	SE	4.30	2.68		4.09	3.50	
PI ³	Mean	6.81	6.80	0.98	6.51	7.08	0.83
	SE	0.55	0.48		0.57	0.79	
Cu, µg/mL	Mean	0.62	0.61	0.66	0.62	0.59	0.83
	SE	0.02	0.02		0.02	0.03	
Zn, µg/mL	Mean	1.01	1.12	0.08	1.08	1.17	0.40
	SE	0.06	0.05		0.08	0.05	
Mn, ng/mL	Mean	1.57	1.86	0.09	1.77	1.94	0.60
	SE	0.15	0.09		0.09	0.14	

¹% Phag = (number of neutrophils with internalized bacteria)/(sum of neutrophils with internalized bacteria and neutrophils without internalized bacteria) $\times$ 100.

²% Killed = (number of dead intracellular bacteria)/(number of dead and live intracellular bacteria) $\times$ 100.

³PI = (number of phagocytized bacteria)/(number of neutrophils with internalized bacteria and neutrophils without internalized bacteria).

Differences in bovine neutrophil function related to supplementation of micronutrients in diets of dairy cows have been demonstrated using the assays employed in the current study (Grasso et al., 1990; Hogan et al., 1990, 1992). Each of these trials reported supplementation of diets or parental injection of Se, vitamin E, or both Se and vitamin E increased intracellular kill of bacteria by neutrophils but had no effect on phagocytic ability of neutrophils. Weiss and Hogan (2005) also investigated neutrophil function differences between cows fed inorganic and organic sources of Se. Neutrophils from cows fed either selenate or Se-yeast supplemented diets did not differ regarding phagocytosis or intracellular kill.

In previous trials intracellular kill by neutrophils was related to the blood concentrations of the micronutrients being tested (Hogan et al., 1990, 1992). However, in the current trial, blood serum concentrations for Cu, Zn, and Mn were not correlated with neutrophil function and did not differ among treatments after 30 d on experimental diets (Table 3). Pearson correlation coefficients between serum mineral concentrations and neutrophil functions on day of neutrophil collection ranged from -0.20 ($P = 0.2$) between serum Mn and phagocytic index to 0.09 ($P = 0.63$) between serum Cu and percentage intracellular kill of bacteria. Normal range for serum Cu is 0.6 to 1.1 $\mu\text{g/mL}$ and serum Zn is 0.6 to 1.9 $\mu\text{g/mL}$ (Herdt and Hoff, 2011). Manganese reference values were estimated as 0.9 to 6.0 ng/mL (Herdt and Hoff, 2011). In the current trial, serum concentrations fell within these normal ranges for Cu, Zn, and Mn in cows fed unsupplemented and supplemented diets. Previous studies found similar results regarding the relationship of unchanging plasma mineral concentrations and neutrophil function when fed organic Cu or Zn (Nemec et al., 2012). Blood concentrations of micronutrients are frequently used in assessment of mineral status of the cow because blood concentrations of minerals are significantly correlated with nutritional status of some trace elements and are more reliable than assessing mineral status from diet evaluation (Herdt and Hoff, 2011).

The lack of change in serum mineral concentrations in the current study during the treatment period may explain the observed similarity among treatments in neutrophil function. Serum concentrations for Cu, Zn, and Mn did not differ among treatments. If no differences were observed among serum values, differences in neutrophil function among treatments were unlikely. Blood values (whole blood, plasma, or serum) for trace minerals are important to measure because they reflect mineral status of transport pools and directly influence mineral availability to neutrophils and other

circulating leukocytes. Neutrophils presumably come in contact and obtaining necessary trace minerals via blood as they are circulating (Leitch et al., 2009). This is especially true for minerals that do not have permeant stores in tissue, such as Zn and Mn. Although cows in the unsupplemented group had a 60-d depletion period, blood values of unsupplemented cows did not differ from those supplemented and were within normal ranges. With no differences among control and supplemented cows in the current study, 60 d during late lactation may not have been long enough to observe differences in serum concentrations for Cu, Zn, and Mn or in vitro neutrophil function. Blood values of Cu, Zn, and Mn indicated the control diet alone was sufficient to maintain adequate concentrations of these minerals for 90 d in late-lactation cows. Blood concentrations of minerals associated with bovine neutrophil function often decrease during the late gestation and periparturient period (Overton and Yasui, 2014). Previous trials determining a positive role of dietary supplementation Cu in improving neutrophil function and reducing mastitis were conducted with cows fed different diets throughout the nonlactating period and early lactation (Scaletti et al., 2003; Siciliano-Jones et al., 2008; Scaletti and Harmon, 2012). The use of late-lactation cows in the current trial may have influenced the effects of dietary supplementation on blood concentrations and neutrophil function differently compared with earlier trials. Although the current trial did not compare cows among different stages of lactation, continued supplementation of Cu, Zn, and Mn during late lactation at concentrations effective at parturition and early lactation may not be warranted.

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