Effect of Copper Depletion and Repletion on Lymphocyte Blastogenesis and Neutrophil Bactericidal Function in Beef Heifers

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ABSTRACT: Thirty-two beef heifers were used to examine the effect of dietary copper depletion and repletion on neutrophil and lymphocyte functions. Heifers allotted to the control group (C+; n = 8) were fed a basal roughage/concentrate diet with Cu-sulfate supplementation (Cu ≥ 8 ppm). To induce a Cu deficiency (depletion phase d 0 to 60), treated (T; n = 24) heifers received a diet supplemented with sulfur (.3 % of diet) and sodium molybdate to achieve a Cu:Mo ratio of 1:1.5. Liver biopsies were collected on d 0, 27, and 60. Despite random allocation, T heifers had lower initial liver Cu concentrations (P < .01) than C+ heifers. At the start of the repletion phase (d 0, equal to d 60 of depletion), treated heifers were allotted by liver Cu concentration to three treatments (n = 8/treatment): Cu sulfate (S; Cu = 10 ppm), Cu proteinate (P; Cu = 10 ppm), or a negative control (C-) that remained on Mo and S supplementation. During the repletion phase, livers were biopsied on d 0, 14, and 45. By d 45, both S and P heifers had greater (P < .05) liver Cu concentrations than C-heifers. For both depletion and repletion phases, no treatment differences were detected in liver Mo or S concentrations. Jugular blood was collected on d 0, 27, and 55 of the depletion phase and d 0, 13, and 42 of the repletion phase. Neutrophils were isolated and incubated with Staphylococcus aureus to determine neutrophil bactericidal capacity (NBC). Lymphocyte blastogenic response (LBR) was monitored during the repletion phase by measuring the amount of [3H] thymidine incorporated when isolated lymphocytes were incubated with pokeweed mitogen, phytohemagglutinin, and concanavalin A. During Cu depletion and repletion, no differences in NBC and LBR were detected. Analysis of whole blood constituents showed no treatment effects for red blood cell count or hematocrit during Cu depletion and repletion. Hemoglobin values were numerically greater for C+ heifers on d 13 and 42 of repletion. These data indicate that the degrees of Cu depletion or repletion achieved in this experiment did not affect neutrophil or lymphocyte function in growing beef heifers.

Key Words: Copper, Neutrophils, Lymphocytes, Heifers

Introduction

Intake of forages high in Mo and adequate in S is considered the leading cause of Cu deficiency in grazing cattle. The production of thiomolybdates occurs when dietary Mo and S combine within the reducing environment of the rumen. These thiomolybdates possess a high affinity for Cu and are capable of rendering it unavailable for absorption (Mason, 1990; Suttle, 1991).

The role of antioxidants in the maintenance of proper immune function has gained considerable attention in human and animal nutrition (Fletcher et al., 1988). Of the microelements, Cu and those metalloenzymes in which it participates seem to have a significant influence on immune function. Recent studies have linked Cu deficiency to reductions in lymphoid organ weights and lymphocyte proliferative responses (Mulhern and Koller, 1988), reduced immunoglobulin M concentrations (Windhauser et al., 1991), and decreases in neutrophil function (Boyne and Arthur, 1986; Xin et al., 1991). The primary objective of this study was to examine the bactericidal capacity of neutrophils and proliferative responses of
lymphocytes from Cu-deficient heifers based on liver Cu concentrations. Furthermore, these responses were followed through a Cu repletion phase in which two lymphocytes from Cu-deficient heifers based on liver biopsy needle (Baxter Healthcare, Valencia, CA). All heifers were fed a basal diet of ground prairie hay (Cu = 1.54 ppm and Mo = .47 ppm; Table 1). Diets were formulated to be isonitrogenous; urea and blood meal were the protein sources. All diets were fed at equal amounts of dry matter to achieve a final heifer weight that would account for 85% of the estimated mature body weight at calving. Copper (8 ppm as Cu sulfate) was supplemented to C+ heifers. To induce a Cu deficiency, T heifers received the basal diet with S added at .3% of the total diet. Sodium molybdate was dissolved in distilled water and sprayed over the concentrate supplement. During the depletion phase, all heifers remained on the same diets. Before Cu repletion, T heifers were reallocated based on liver Cu concentrations to one of three treatments (n = 8/treatment), Cu sulfate (S; Cu = 10 ppm), Cu proteinate (Nutribasics, Highland, IL; P; Cu = 10 ppm), or a negative control (C-) that remained on the previous Cu depletion diet consisting of Mo and S supplementation.

Liver Biopsies and Analyses. On d 0, 27, and 60 of the depletion phase and d 13 and 42 of the repletion phase. Following collection, blood was centrifuged at 800 x g for 20 min, and plasma was harvested. Roswell Park Memorial Institute (RPMI; Gibco Laboratories, Grand Island, NY) 1640 medium was then added and gently mixed, returning each sample to the original volume of 30 mL. The mixture was then layered onto 15 mL of a density gradient solution (Histopaque-1077; Sigma Diagnostics, St. Louis, MO). Samples were centrifuged at room temperature for 40 min at 800 x g to separate mononuclear cells over the density gradient. The mononuclear cell band was collected for a lymphocyte blastogenic assay. Approximately one-third of the packed red blood cells were discarded, and the remaining pellet was resuspended in cold .2% NaCl for 30 s. Isotonicity was restored after the addition of an equal volume of 1.6% NaCl for 30 s. These washes were repeated three to four times to ensure the lysis of red blood cells. After washing, neutrophils were counted using an electronic cell counter (Danam Electronics, Dallas, TX). Final cell dilutions of 1 x 10^7 cells/mL were made with RPMI 1640 supplemented with 5% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT) and without antibiotics.

Neutrophil Bactericidal Activity. Bactericidal activity was determined with procedures described by Stevens et al. (1991) with slight modifications. Briefly, neutrophils (50 µL, 1 x 10^7 cells/mL) were incubated at 37°C for 1 h with opsonized Staphylococcus aureus (50 µL, 1 x 10^9 cells/mL). The S. aureus (1 x 10^9 bacteria/mL) was opsonized with bovine anti-S. aureus serum (1:10) at room temperature for 30 min. Controls consisting of neutrophils and medium or S. aureus and medium also were plated. After incubation, neutrophils were lysed with the addition of 50 µL of .15% saponin. Next, 50 µL of 3-[4,5-dimethylthiazol-2-ol]-2,5-diphenyltetrazolium bromide (MTT; Sigma) was added to all wells and incubated for 10 min at room temperature. After the plates were centrifuged for 5 min, well contents were removed by blotting onto an absorbent pad, and 150 µL of acid isopropanol (.04 N HCl) was added to each well for 10 min. Plates

Table 1. Nutrient content of prairie hay

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Nutrient analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>5.00 %</td>
</tr>
<tr>
<td>Calcium</td>
<td>5,448.3 ppm</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>780 ppm</td>
</tr>
<tr>
<td>Copper</td>
<td>1.54 ppm</td>
</tr>
<tr>
<td>Molybdenum</td>
<td>47 ppm</td>
</tr>
<tr>
<td>Zinc</td>
<td>16.63 ppm</td>
</tr>
<tr>
<td>Manganese</td>
<td>28.30 ppm</td>
</tr>
</tbody>
</table>

*All diets were fed at equal amounts of dry matter to achieve a final heifer weight that would account for 85% of the estimated mature body weight at calving.
were agitated for 5 min, wrapped in parafilm and foil, and stored in the dark at 4°C overnight. Crystallin contents were dissolved by adding 50 µL of PBS to each well, and plates were agitated until the crystals dissolved. Well contents were transferred to a new plate, and absorbance was measured at a wavelength of 570 nm (Bio-Tek Inst., Winooski, VT). A corrected optical density (OD) for each sample was calculated by subtracting a negative control (neutrophils and media) value from the sample value. The percentage of bacteria killed was determined as 1 - [(corrected OD of sample) - (OD at 90% killing)] / [(OD at 0% killing) - (OD at 90% killing)] × 90%.

Lymphocyte Blastogenic Response. On d 0, 13, and 42 of the Cu repletion phase, the blastogenic response of isolated lymphocytes to mitogens was evaluated. During steps for neutrophil isolation, mononuclear cells (> 95% lymphocytes) were collected from the buffy coat following density gradient centrifugation. Cells were resuspended with 10 mL of RPMI 1640 and centrifuged at room temperature for 10 min at 800 x g. Cells were washed with this procedure an additional two times. Lymphocyte concentrations were determined, and final dilutions were adjusted to 3 x 10^6 cells/mL by adding RPMI 1640 culture medium containing 10% FBS, 1% antibiotic-antimycotic (10,000 units/mL of penicillin G; Gibco Laboratories), and 1% Fungizone (275 µg/mL of amphotericin-B; Gibco Laboratories). Lymphocyte blastogenesis was carried out with procedures described previously (Blecha et al., 1984). The assay used 100 µL of isolated, washed lymphocytes (3 x 10^6 cells/mL), which were added to wells in a sterile, 96-well, round-bottom microtiter plate. Phytohemagglutinin (PHA; Wellcome Diagnostics, Dartford, U.K.), pokeweed mitogen (PWM; Gibco Laboratories), and concanavalin A (ConA; Pharmacia Laboratories, Piscataway, NJ) were rehydrated as prescribed (PHA, 10 µg/mL; PWM, 1:10 ratio; and ConA, 20 µg/mL). Mitogens were resuspended in RPMI 1640 with 10% FBS and antibiotics and added (100 µL) to triplicate lymphocyte cultures. Unstimulated cultures containing culture medium and lymphocytes acted as controls. Cultures were incubated at 37°C in a humidified 93% air, 7% CO₂ environment for 66 h. Lymphocyte blastogenic response was determined by the incorporation of 20 µL of .05 µCi/µL of [³H] thymidine (methyl [³H] thymidine, specific activity = 44 Ci/mM, Amersham Life Science, Buckinghamshire, U.K.) for 18 h before cells were harvested. Cells were harvested onto filter paper with deionized water by an automated cell harvester (PHD Cell Harvesting System, Model 200, Cambridge Technology, Cambridge, MA). Results were expressed as net counts/minute (counts per minute of mitogen-stimulated cultures minus counts per minute of unstimulated cultures). The average CV for the log counts per minute of mitogen-stimulated lymphocytes was 6.4 ± 4.4. The same lots of FBS, RPMI 1640 culture medium, and PHA, PWM, and ConA mitogens were used throughout the experiment to decrease assay variability.

Whole Blood Constituents. Jugular blood (5 mL) was collected into tubes containing EDTA on d 0, 27, and 55 of the depletion phase and d 13 and 42 of the repletion phase. Analyses of whole blood, including hematocrit (HCT), hemoglobin (HGB), and red blood cell counts (RBC), were made using the Danam Cell Counter.

Statistical Analyses. For the depletion phase, heifers were allotted by weight to pens of equal size. Before Cu repletion, heifers were reallocated by liver Cu concentration. A split-plot design was used with pen serving as the whole plot and time as the subplot. Treatments were allocated randomly to pens. Analysis of variance was performed using the GLM procedures of SAS (1985). When there were time x treatment interactions, treatment means within times were compared using least significant differences, calculated using the appropriate computed standard error.

Results and Discussion

Heifer Weight Change. Treatment did not affect weight gain during the depletion (.86 ± .06 and .92 ± .12 kg heifer⁻¹ d⁻¹ for C- and C+, respectively) or repletion (.74 ± .08, .71 ± .01, .79 ± .12, and .79 ± .14 kg heifer⁻¹ d⁻¹ for C-, S, P, and C+, respectively) phases of this experiment. Other investigators reported decreases in weight gain of cattle consuming high-Mo diets (Boyne and Arthur, 1986; Philippo et al., 1987b). In contrast, Xin et al. (1991) did not report any differences in weight gain of steers consuming Mo-supplemented diets similar to those used by Philippo et al. (1987b). Stednick et al. (1985) reported increases in weight gain for cattle that were diagnosed as Cu-deficient and subsequently supplemented with increased amounts of Cu sulfate. Arthington et al. (1992) reported significant reductions in the weaning weight of Cu-supplemented calves, via boluses containing Cu oxide, compared with non-bolused calves. The wide variation in weight gain during Cu deficiency or Cu repletion probably is explained best by the intricate interaction of all trace elements. In such situations, controlling the dietary intake of all minerals is nearly impossible; therefore, each diet is unique and the impact that it exerts on various production variables may vary.

Liver Mineral Concentrations During the Depletion Phase. Following random allocation of heifers to treatment, C+ heifers had greater (P < .01) initial liver Cu concentrations. Treated heifers experienced continued decreased liver Cu concentrations, whereas C+ heifers had a continuous increase (Figure 1). This difference was most dramatic (P < .01) by d 60, and at this time T heifers were considered nearly Cu-deficient (Cu = 38.8 ppm) according to the concentrations described by Puls (1988) in which Cu deficiency...
Figure 1. Liver copper concentrations in control (C+) and Mo- and S-supplemented heifers (C-) during the Cu depletion phase. Treatment means differ (P < .01) for each day (SEM = 3.5 and 6.2 ppm for C- and C+, respectively). On d 60 C- heifers were considered at or near Cu deficient (liver Cu = 38.9 ppm).

is defined as liver Cu concentrations ≤ 35 ppm. The decrease in liver Cu when heifers were supplemented with sodium molybdate agrees with results of other studies (Thomas and Moss, 1951; Phillippo et al., 1987b). The latter authors used similar sodium molybdate supplementation with more dramatic results; after d 56 of treatment, liver Cu concentrations decreased from >120 to <20 ppm. However, these investigators used young calves that were fed a low-Cu diet before the start of the trial. This early Cu deprivation may have predisposed the calves to a more rapid onset of Cu deficiency. Thomas and Moss (1951) fed greater amounts of sodium molybdate (>250 ppm) and, consequently, classic symptoms of Cu deficiency, such as greying of black hair and lameness, appeared after 42 d of treatment. These results indicate that basal diet, level of Mo supplementation, and animal age may all play roles in the impact of Mo on Cu depletion.

Neither liver Mo nor S concentration differed during the depletion phase. In another study (Xin et al., 1991), steers were fed ammonium molybdate (10 ppm) to create a Cu deficiency. In this study, there was a gradual accumulation of liver Mo over an 8-mo treatment period. This indicates an excess of Mo, more than that which can combine with available S to form thiomolybdates (Mason, 1990). In such instances, S may be the limiting element in the development of a Cu deficiency (Suttle, 1975).

Liver Mineral Concentrations During the Repletion Phase. Before the initiation of repletion, C-, P, and S heifers did not differ (P > .90) in liver Cu concentrations (Table 2). However, the three groups had lower (P < .05) liver Cu concentrations than C+ heifers. By d 14, S- and P-supplemented heifers had numeric increases in liver Cu; however, these values were lower (P < .05) than those of C+ heifers. By d 45 of Cu repletion, S and P heifers had greater (P < .05) liver Cu concentrations than C- heifers. In C- heifers, values remained lower (P < .05) than those of C+ heifers because of their gradual and continual increase in liver Cu concentration throughout the trial. The use of tissue uptake of minerals as an estimate of bioavailability is an effective means of comparing various sources of a common mineral (Ammerman et al., 1985). The overall magnitudes of response to Cu supplementation for the repletion phase, as measured by liver Cu, were 48.9 and 67.7 ppm for S- and P-supplemented heifers, respectively; however, this difference was not significant (P = .27). No treatment differences in liver Mo or S concentrations were detected during the repletion phase. Kincaid et al. (1986) reported an increased relative availability of Cu from proteinate vs sulfate sources, whereas Wittenberg et al. (1990) did not report a difference in Cu

Table 2. Effect of copper repletion on liver copper concentrations

<table>
<thead>
<tr>
<th>Day</th>
<th>C-</th>
<th>S</th>
<th>P</th>
<th>C+</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>37.7g</td>
<td>40.0g</td>
<td>38.9g</td>
<td>127.7h</td>
<td>6.0</td>
</tr>
<tr>
<td>14</td>
<td>27.9g</td>
<td>49.1g</td>
<td>56.3g</td>
<td>129.2h</td>
<td>6.0</td>
</tr>
<tr>
<td>45</td>
<td>39.8g</td>
<td>88.9h</td>
<td>106.6h</td>
<td>193.0i</td>
<td>6.0</td>
</tr>
</tbody>
</table>

aValues are expressed as ppm on a dry matter basis.

bBasal diet supplemented with Mo (Cu:Mo = 1:1.5) and 8 (.3% of diet).

cBasal diet supplemented with copper sulfate at 10 ppm.

dBasal diet supplemented with copper proteinate at 10 ppm.

eBasal diet supplemented with copper sulfate at 8 ppm.

fSEM has been pooled for all means.

g,h,iMeans within day differ (P < .05).
feeding hay naturally high in compared while animals were under the influence of investigated the role of organic (Cu lysine) vs dietary (Cu proteinate and Cu sulfate availability. However, these two trials represent some important differences between themselves and the current trial. Kincaid et al. (1986) used young calves and induced a Cu deficiency with ammonium molybdate. Wittenberg et al. (1990) used older steers and induced a Cu deficiency by feeding hay naturally high in Mo. In both cases, the efficacy of Cu proteinate relative to Cu sulfate was compared while animals were under the influence of dietary Mo supplementation. The current study compared Cu sources by removing Mo supplementation and repleting Cu-deficient heifers that were consuming the basal diet only. Nockels et al. (1993) investigated the role of organic (Cu lysine) vs inorganic (Cu sulfate) Cu sources in stressed calves. Their results showed a 62% greater retention of Cu lysine compared with Cu sulfate and support the use of organic Cu during instances when an increased availability is desired, such as during situations of stress or illness.

Neutrophil Bactericidal Capacity. No treatment differences were detected in neutrophil bactericidal capacity for any of the sampling periods during the depletion (Table 3) and repletion phases of the experiment. These data are in contrast to those reported by other investigators (Boyne and Arthur, 1986; Xin et al., 1991) in which similar Cu-depletion diets were fed. Variations in these results may be attributable to differences in the magnitude of Cu deficiency at the time of sampling. However, by criteria outlined by Puls (1988), animals in each of these three trials were classified as marginal to deficient in Cu. Other explanations could be the duration of supplementation and the form of Mo supplemented. Boyne and Arthur (1986) and Xin et al. (1991) used ammonium molybdate for ≥ 6 mo, in comparison to the current study in which sodium molybdate was supplemented for 2 mo. Another interesting point to consider is the influence of Mo acting independently of Cu to affect neutrophil function. Molybdenum, although highly effective in decreasing liver Cu stores, also may have direct effects on the physiology of the animal (Phillippo et al., 1987a). Xin et al. (1991) reported substantial increases in liver Mo after 30 d of ammonium molybdate supplementation. Liver Mo in the current study was not increased by sodium molybdate supplementation.

Lymphocyte Blastogenic Response. No differences in lymphocyte blastogenesis following mitogen stimulation were detected at the onset or conclusion of the repletion phase. These data are in agreement with those of Stabel et al. (1993), who also reported no copper effect on mitogen-stimulated bovine lymphocyte proliferation. In contrast, however, they reported marked increases in mitogen-induced proliferation of lymphocyte cultures when supplemented with Cu chloride. Our in vivo data did not reflect these results when Cu-deficient heifers were repleted. Ward et al. (1992) reported similar results in lymphocyte function when calves were supplemented with Mo and either Cu-lysine or Cu-sulfate sources. These data are in partial agreement with the results with mitogen-stimulated porcine lymphocytes (Baia et al., 1992). In an earlier study, Baia et al. (1991) reported significant decreases in the ability of lymphocytes from Cu-deficient male rats to respond to stimulation from PHA, ConA, and LPS mitogens. In contrast, Windhauser et al. (1991) did not find significant differences in the blastogenic abilities of lymphocytes from Cu-deficient rats stimulated with PHA, PWM, and ConA. Our data indicate that lymphocyte function was not compromised even when liver Cu differed by nearly fivefold (C− vs C+ at d 42).

Table 3. Effect of copper depletion on neutrophil bactericidal activitya

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>C−</th>
<th>C+d</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25.3 ± 3.1</td>
<td>26.4 ± 5.4</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>27.8 ± 3.1</td>
<td>28.8 ± 5.4</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>30.9 ± 3.2</td>
<td>38.2 ± 5.8</td>
<td></td>
</tr>
</tbody>
</table>

aPercentage of Staphylococcus aureus killed ± SE.

bNeither the time × treatment interaction nor treatment effects were significant; therefore, no individual mean comparisons were made.

cBasal diet supplemented with Mo (Cu:Mo = 1:1.5) and S (.3% of diet).

dBasal diet supplemented with copper sulfate at 8 ppm.

Table 4. Effect of copper repletion on whole blood measurements of hemoglobina

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>C−</th>
<th>S</th>
<th>P</th>
<th>C+d</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13.50f</td>
<td>12.79f</td>
<td>12.85f</td>
<td>12.86f</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>12.95fg</td>
<td>11.81fg</td>
<td>12.96fg</td>
<td>13.18fg</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>12.61fg</td>
<td>12.24fg</td>
<td>12.08fg</td>
<td>13.38fg</td>
<td></td>
</tr>
</tbody>
</table>

aValues are expressed as grams/deciliter. The overall SEM was .35 g/dL.

bBasal diet supplemented with Mo (Cu:Mo = 1:1.5) and S (.3% of diet).

cBasal diet supplemented with copper sulfate at 8 ppm.

dBasal diet supplemented with copper proteinate at 10 ppm.

fMeans with unlike superscripts within day differ (P < .1)
Whole Blood Constituents. Before Cu repletion, red blood cell count and hematocrit were not different. Hemoglobin values were numerically greater for the C+ heifers on d 13 and 42; however, this difference was not significant. Additionally, no differences within treatment were detected from d 0 to 42 of repletion (Table 4). These results are in agreement with Blakemore and Venn (1950) and within the normal range for cattle (Smart and Gudmundson, 1980). In contrast to the current study, Sanders and Sanders (1983) reported low hemoglobin (6 to 8 g/dL) and hematocrit (18 to 27%) values for a dairy herd that was diagnosed as Cu-deficient. However, the diagnosis of Cu deficiency in that study may be questionable, because no estimates of Cu status in the animals were evaluated. The investigators relied on mineral evaluation of the diet, which was reported to be Cu-deficient. Nevertheless, production increased after the diets were fortified with Cu. Jones and Suttle (1981) did not find a reduction in hemoglobin concentrations in sheep diagnosed as Cu-deficient. Similarly, Bala et al. (1992) did not report a decreased hematocrit in Cu-deficient pigs.

Implications

Dietary molybdenum and sulfur can significantly influence the metabolism of Cu in ruminants. In the repletion phase of this experiment, no differences in liver copper were detected when copper proteinate vs copper sulfate was used as the dietary source. Additionally, immune competence, as measured by neutrophil bactericidal activity and lymphocyte blastogenesis, did not seem to be affected by copper deficiency at the degree and duration achieved in this experiment. Field and case studies continue to indicate improvements in overall herd health when animals are supplemented with increased dietary copper. Therefore, continued investigation in the area of dietary copper and immune function merits attention.

Literature Cited


Performance and immune response in growing cattle fed molybdenum and different copper levels and sources. J. Anim Sci. 70(Suppl. 1):32 (Abstr.).

